POSSIBLE ROLE OF CALCIUM IN THE MECHANO-ELECTRIC TRANSDUCTION PROCESS OF MAMMALIAN SLOWLY ADAPTING TYPE I MECHANORECEPTORS IN THE SKIN

BY

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CONTENT

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ABSTRACT

A mammalian isolated skin-nerve preparation was adapted to study responses of slowly adapting type I (SA I) and slowly adapting type II (SA II) mechanoreceptors in the skin. Standard mechanical stimuli of usually 15 mN constant force were applied every 30 seconds while single unit recordings were made from the cutaneous nerve supplying the skin. Drugs interfering with the flux of calcium ions through cell membranes were applied in order to examine the hypothesis of synaptic transmission between Merkel cell and afferent nerve fibre in SA I receptors believed to require pre-synaptic calcium influx and to compare the effects with those on SA II receptors (Ruffini corpuscles) known not to involve synaptic transmission.

The responsiveness of the receptors in the in-vitro preparation was found to be stable over several hours. Their discharge rates and firing pattern compared well with those found in studies in situ.

Neomycin affected the mechano-transduction process of both SA I and SA II receptors, decreasing the rate of firing. The stronger suppression of SA I than SA II receptors, found in previous studies in situ, was only observed at a concentration of 0.5 mM but not at 5 mM. This may be a result of neomycin interfering with calcium currents through mechano-electric transduction channels.

Mg^{++} ions depressed both SA I and SA II receptors to a similar degree. This effects was enhanced rather than antagonised by increase of the calcium concentration in the solution. Thus, the general stability of the membrane was probably affected through the screening of negative charges.

Verapamil had no effect in concentrations up to 10 μM, but when applied in high concentration (100 μM), produced a non-specific partial nerve block while the mechano-transduction process appeared not to be affected.

The calcium channel agonist Bay K 8644 was found to increase the firing rate of the SA I as well as SA II receptors in a dose dependent manner.

Involvement of intracellular calcium stores was studied with caffeine which may stimulate calcium release from the sarcoplasmic reticulum. In some tissues it is also known to increase intracellular cAMP and thereby opening L-type calcium channels. Application of caffeine, IBMX and 8-Bromo-cAMP stimulated the responses of both type I and type II receptors suggesting a cAMP mediated rather than a direct effect of caffeine.

Sodium azide has been found to block intracellular calcium uptake into mitochondria. In the present experiments it inhibited responses of SA I receptors but stimulated those of SA II receptors.

These results indicate that calcium ions play a vital role in the normal function of both SA I and SA II receptors. No qualitative differences were found for the different substances interfering with calcium influx into the two types of receptor. Thus, the present study did not provide evidence for a specific pre-synaptic calcium influx into Merkel cells which would support the hypothesis of synaptic transmission between Merkel cell and afferent nerve fibre. However, completely different responses between SA I and SA II receptors could be seen while interfering with intracellular calcium sequestration e.g. using sodium azide. Further studies will be required to elucidate the role of intracellular calcium stores in these receptors.

INTRODUCTION

The structure and functional characteristic of the mammalian slowly adapting type I (SA I) receptor were first described by Merkel in 1875. They are located at the epidermo-dermal border (Andres & v.Düring, 1973) and consist of clusters of special cells - "Merkel cells" - in close apposition to an enlarged terminal branch of the myelinated afferent nerve fibre. Covered by the epidermis and shielded from below by the chorium, they are virtually inaccessible for microelectrode recordings. In consequence, the mechanism of the mechano-electric transduction and the role of the Merkel cells are still controversial. Under the electron-microscope structures can be seen suggesting that a chemical synapse exists between Merkel cell and nerve terminal (Iggo & Andres, 1982; Iggo & Findlater, 1984). The hypothesis of synaptic transmission gained further support from the unique irregular firing pattern of SA I receptors which could best be explained by the existence of independent pacemakers which are not reset by the generation of an action potential in the nerve fibre (Horch et al., 1974). Met-enkephalin like substances have been described immuno-histochemically in Merkel cells and were suspected to act as neuro-transmitter (Hartschuh et al., 1979; Cheng Chew & Leung, 1991). However, the classical opioid receptor antagonist naloxone had no effect on responses of sinus hair type I receptors also employing Merkel cells (Gottschaldt & Vahle-Hinz, 1982). Thus, so far there has been no direct proof for the hypothesis of synaptic transmission and no transmitter substance has yet been clearly identified.

The requirement of calcium entry via the voltage gated calcium channels for transmitter release is well established in all mammalian synapses studied so far (for review see Augustine et al., 1987). Calcium currents are also found to be a major component in the mechano-sensitive current in inner ear hair cells. The hair cell is a well studied mechano-transduction system and is known to transmit its signals to the afferent nerve through a chemical synapse (for review see Ashmore, 1991). Based on similarities between inner ear hair cells and Merkel cell receptors, analogue functioning of the two receptor types had been suggested (Iggo & Findlater, 1984).

Aminoglycoside antibiotics like neomycin are known to acutely impair the function of hair cells in the inner ear by interfering with calcium currents (Ohmori, 1985; Kroese et al., 1989). In previous studies in this laboratory, neomycin was found to reduce responses of SA I receptors (Baumann et al., 1990) believed to be the result of blocking of calcium channels similar to results obtained with calcium channel blocking agents (Pacitti & Findlater, 1988).

The present study aimed to examine the role of calcium ions in the function of the SA I receptors and their possible involvement in synaptic transmission by comparing effects on receptor responses with those obtained under the same conditions from slowly adapting type II (SA II) receptors. For the latter type of receptor (Ruffini corpuscles) it is well established that the mechano-electric transduction process occurs directly in the fine terminal branches of the nerve fibre and does not involve synaptic transmission (Chambers et al., 1972). In order to modulate transmembrane calcium currents, ions like Mg^{++} known to compete with Ca^{++} at the channels, the classic organic calcium channel blocker

verapamil and the channel activator BAY K 8644 were chosen as test substances. In addition some representatives of drugs known to affect the release from or uptake into intracellular calcium stores were tested as another means of changing intracellular free calcium concentration. In order to allow localised application of the drugs only to the immediate vicinity of the receptor and avoiding the risk of interfering with blood flow and nerve conduction or affecting the visco-elastic properties of the skin, an isolated skin-nerve preparation originally developed by Reeh (1986) and adapted for experiments on mechano-receptors in this laboratory was chosen as experimental model.

SECTION 1. REVIEW

1.1 Sensory endings in mammalian skin

The mammalian skin is rich in sensory innervation. Sensory afferents can be functionally classified by their specialised responses to various environmental stimuli such as heat, cold and touch. Those which respond to mechanical force such as vibration, indentation and stretching of the skin are called mechanoreceptors. These include the Pacinian corpuscle, the rapidly adapting mechanoreceptors, the slowly adapting type I and slowly adapting type II receptors. The morphology and functional characteristics of some of these receptors are summarised in Fig. 1.1. The Pacinian corpuscles are very sensitive vibration sensors which respond to high frequency vibrations in the skin. The rapidly adapting mechanoreceptors such as the Meissner corpuscle, the hair follicle or Krause endings respond only to the onset of a stimulus and adapt rapidly. Only the two types of slowly adapting mechanoreceptors, Ruffini corpuscle and the Merkel cell receptor respond to slow and maintained mechanical indentation of the skin with sustained discharge in the afferent nerve fibre (Iggo & Muir, 1969; Chambers et al., 1972).

1.2 The Slowly Adapting Type I mechanoreceptor

The SA I receptor which can be seen as a dome structure in the hairy skin of some mammals consists of a single layer of Merkel cells each in close contact with an enlarged terminal branch of the afferent nerve fibre (Fig. 1.2a). On the epidermal side, Merkel cells have a number of finger like cytoplasmic projections reaching into the adjacent keratinocytes. The Merkel cell is characterised by a lobulated nucleus and large number of

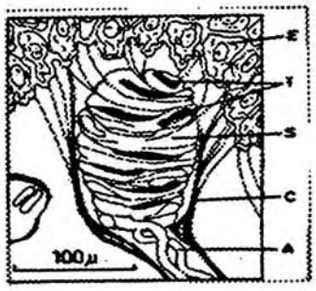
dense core granules on the basal side (Iggo & Muir, 1969). The function of these granules is still unknown but various substances have been found in these granules. They exhibit immunoreactivity with met-enkephaline like peptides (Hartschuh et al., 1979, Cheng Chew & Leung, 1991) which may act as neurotransmitters or neuromodulators. Other structures particular to neural secretory cells like a prominent Golgi apparatus and special junctional areas are also found in the Merkel cell (Iggo & Muir, 1969; Andres & v.Düring, 1973).

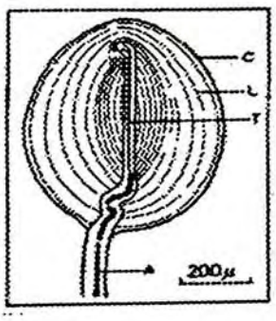
Physiologically the SA I receptor is characterised by its irregular pattern of firing in response to sustained mechanical stimuli (Horch et al., 1974). Fig. 1.2b compares the firing pattern of the SA I and SA II receptors. This irregular firing pattern was important in identifying the SA I receptors during experiments.

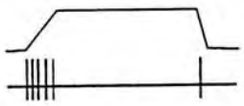
1.3 The Slowly Adapting Type II mechanoreceptor

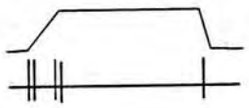
The slowly adapting type II mechanoreceptor or the Ruffini corpuscle is located deep in the dermis and is characterised by its regular firing pattern in response to sustained mechanical stimulation. Unlike the type I receptor, it is also responsive to stretching of the skin. Histologically, it belongs to the type of encapsulated sensory endings which has a fibrous sheath surrounding the branched endings of the afferent nerve fibre. There are no secondary cells like the Merkel cells in these receptors. Action potentials are thought to originate directly from the branches of the nerve fibre (Chambers et al., 1972).

Rapidly Adapting





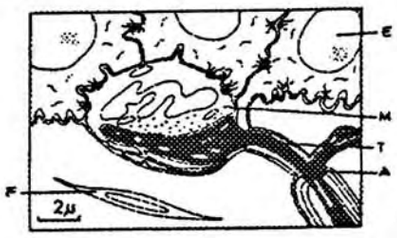


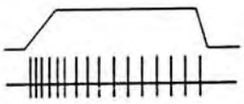


Meissner endings

Pacinian endings

Slowly Adapting





Merkel endings

Ruffini endings

Fig 1.1 Morphology and functional characteristics of various mechanoreceptors in the skin. For details see Emslie-Smith et al, (1988)

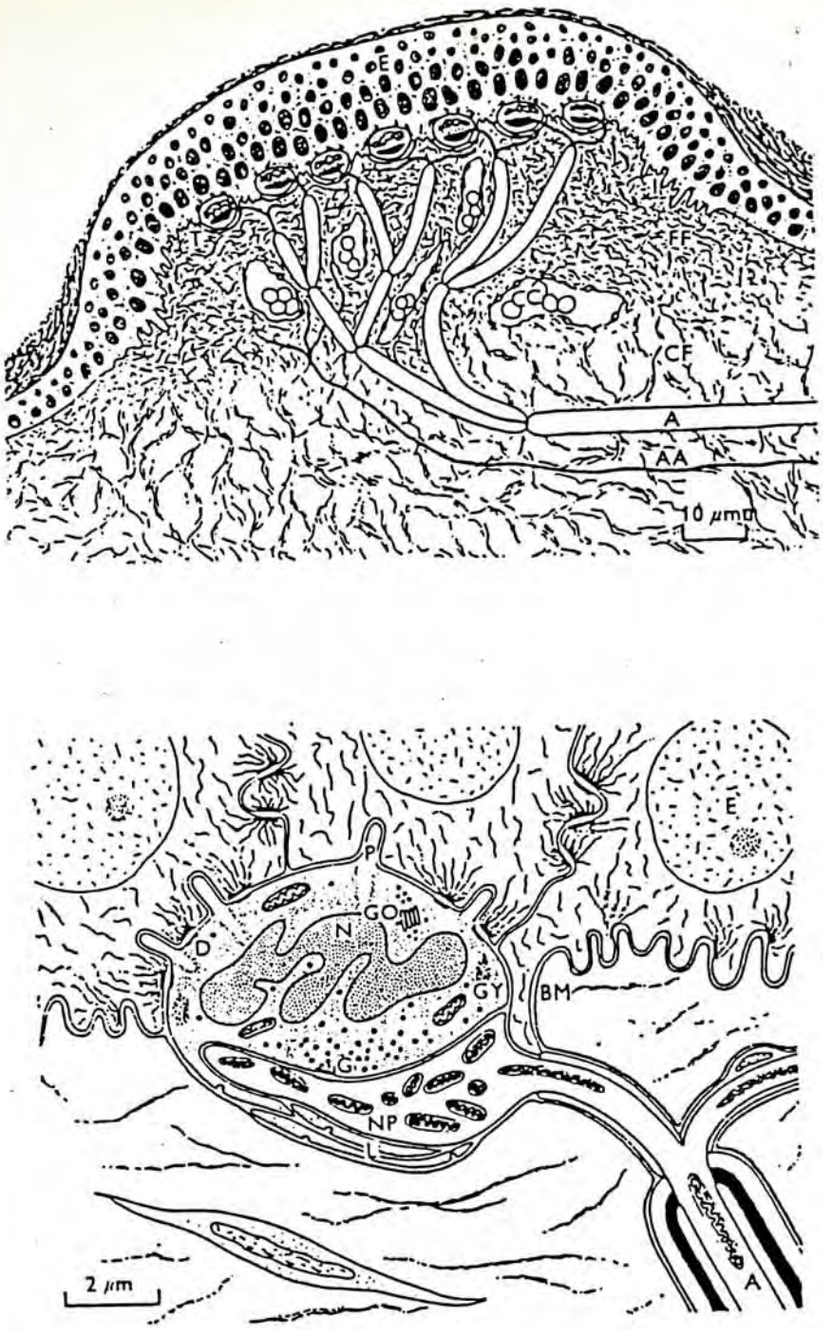


Fig. 1.2 Morphology of the touch dome (SAI receptor, upper graph), and the relation between the Merkel cell and the nerve terminal (lower graph). (from Iggo & Muir, 1969)

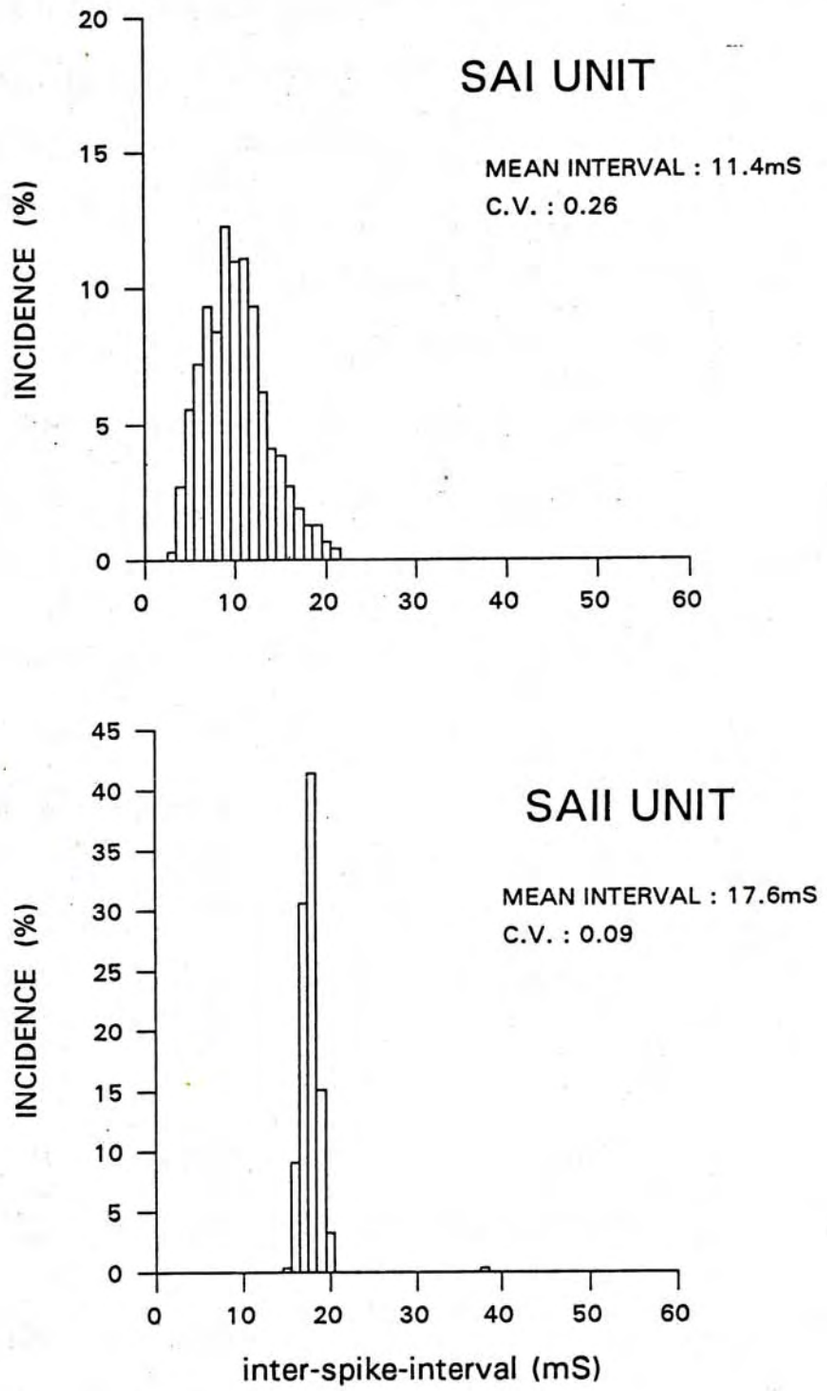


Fig. 1.2b Typical firing patterns of SAI and SAII receptors .

1.4 The role of the Merkel cell

The role of the Merkel cell has been studied by various investigators, for review see Iggo & Findlater (1984). Based on morphological and functional information, the authors arrived at the hypothesis that the Merkel cell is a transducer cell responding to mechanical forces in the skin by secreting a chemical transmitter substance to the enlarged terminal of the afferent nerve (Iggo & Muir, 1969; Horch et al., 1974). Others dispute the existence of a chemical synapse and argue that the Merkel cell has only a supportive function and the actual transduction occurs at the nerve (Smith, 1977; Gottschaldt & Vahle-Hinz, 1981; Diamond et al., 1988).

The evidence that favours a mechano-transduction and neurosecretory role of the Merkel cell include:

1. Specialised junctional morphology suggestive of a synapse as revealed in ultrastructural studies (Chen et al., 1973; Byers & Costello, 1988).

2. The existence of putative neurotransmitters inside the Merkel cell (Hartschuh et al., 1979 and 1983; Cheng Chew & Leung, 1991).

3. The unique discharge pattern of SA I receptors. The most likely mechanism to produce such an irregular pattern is the release of transmitter from the Merkel cells (Horch et al., 1974).

4. Failure of the SA I receptor to respond to mechanical stimuli under hypoxic conditions within a short period of time while the afferent nerve can still be electrically excited to conduct action potentials (Iggo & Findlater, 1984).

5. Depletion of dense core granules in the Merkel cell after receptor failure under hypoxic condition (Iggo & Findlater, 1984).

On the other hand, the following arguments have been put forward against a mechano-transducton and neurosecretory role of the Merkel cell.

1. The putative synaptic junctions were by no means typical compared to other well established chemical synapses (Diamond et al., 1988).

2. No drugs were found that could produce spontaneous action potentials in the afferent fibre innervating SA I receptors (Smith and Creech, 1967).

3. SA I receptors were still mechanosensitive after the Merkel cells had been destroyed (Diamond et al., 1988).

4. After cutting the afferent nerve fibre regenerating axons grow preferentially towards old receptor sites (Horch, 1982). This suggested that the Merkel cell may have a role as the target for the afferent fibre, an idea originally proposed by Scott et al., (1981).

1.5 The Hair cell as an analogy

The vestibular hair cell of the inner ear is a simple and well studied mechano-electric transduction system which relays its signal to the afferent nerve via a chemical synapse (Corey & Hudspeth, 1979; Akeov & Andrianov, 1989). The sensory hair or stereocilium at the apex of the cell is a projection of the cell membrane. Bending of the hair is the first

step in the excitation process of this transducer cell (Fig. 1.3). The actin filaments similar to those running down the length of each stereocilium are also found in the cytoplasmic projections of the Merkel cell (Iggo & Muir, 1969). It has now been found that both the mechano-electric transduction at the apex of the vestibular hair cell and the mechanism for its transmitter release are calcium dependent (Hudspeth, 1982; Ohmori, 1985; Chabbert et al., 1991).

1.6 Molecular mechanism of mechano-electrical transduction

To further understand the role of the Merkel cell and mechano-electric transduction in general, Iggo & Findlater (1984) had proposed, in analogy to the situation in the vestibular hair cells, a hypothesis of sequence of events happening when the SA I receptor is stimulated, as follows (Fig. 1.4). 1.) Mechanical distortion of filamentous rods of Merkel cells, 2.) alteration of membrane permeability of the epidermal surface of Merkel cell leading to 3) entrance of Ca ions into the Merkel cell, 4.) mobilisation of osmiophillic granules, 5.) release of granule contents at the synapse-like junction between Merkel cell and nerve plate, 6.) alteration in membrane permeability of the nerve plate membrane leading to the 7.) development of a generator potential with the consequent 8.) initiation of an action potential in the myelinated afferent fibre. The present study concentrated on those stages involving calcium ions.

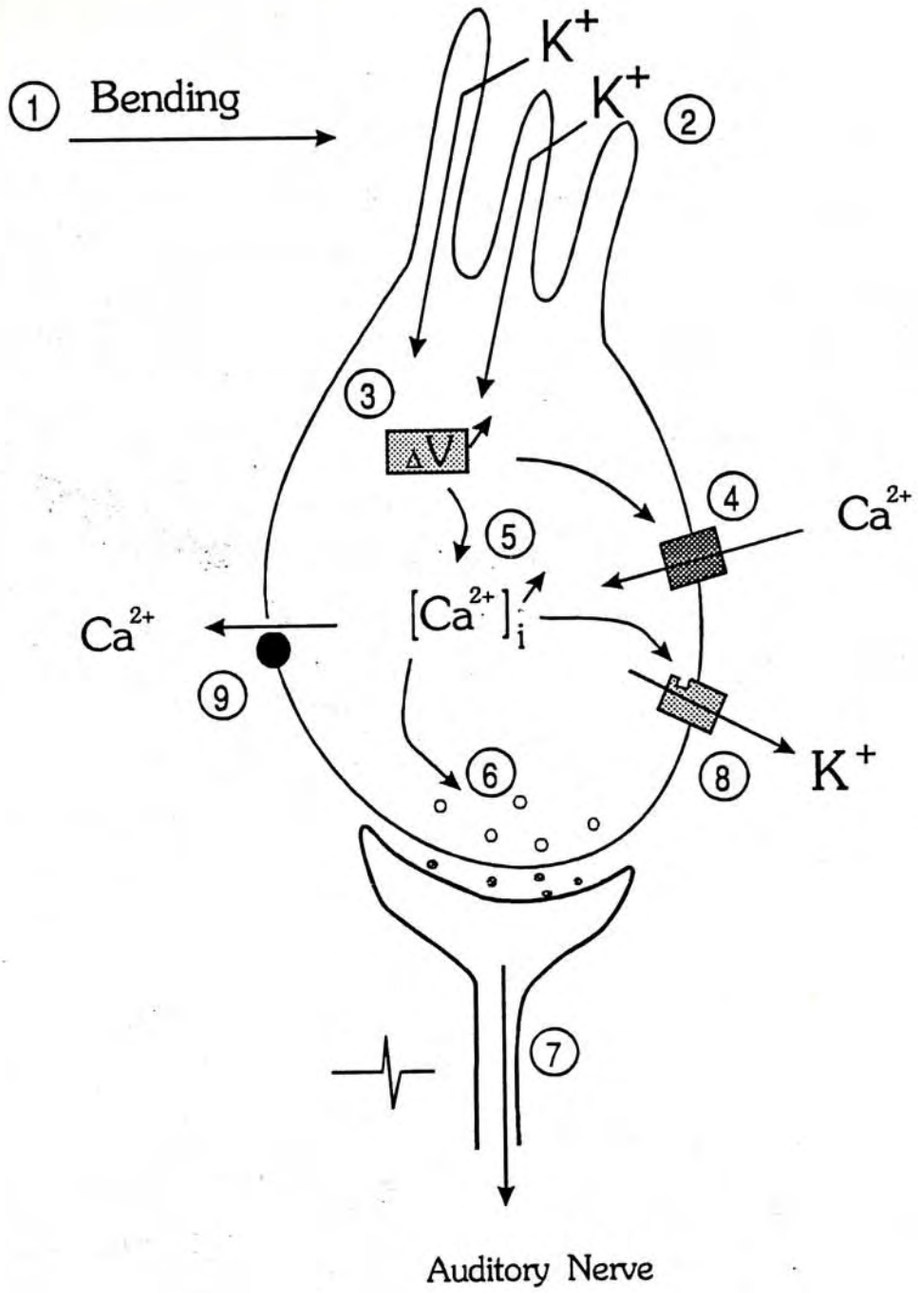


Fig 1.3 Schematic diagram of the mechanotransduction mechanism of vestibular hair cell. (modified and redrawn after Akeov & Adrianov (1989) and Dulon & Aran (1990).

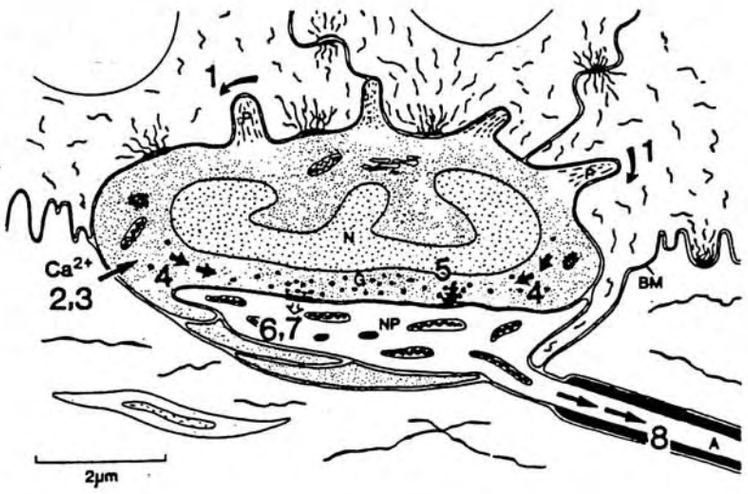


Fig 1.4 Merkel cell-neurite complex showing the suggested sequence of events in the proposed transduction process. See text for detail. (from Iggo & Findlater, 1984)

1.7 Calcium influx and transmitter release

Classical experiment by Katz & Meledi (1970) had established the requirement of Ca influx for transmitter release. In neurons, Ca^{++} enters the cytoplasm from the extracellular milieu through voltage-gated calcium channels. Three kinds of calcium channels have been characterised, namely the L-, N-, and T-type channels. They each have distinctive characteristics like single channel conductance, activation kinetics and sensitivity to pharmacological substances (reviewed by Tsien et al., 1988, Augustine et al., 1987). Different channels may appear in different tissues and have different functions. The secretion of neurotransmitter substances from dense-cored granules is now thought to follow the Ca^{++} influx through the high-conductance, slow-inactivation L-type channels (Trimble et al., 1991). This type of channel has just been found in isolated rat Merkel cells (Yamashita et al., 1991), although their physiological function in the Merkel cell has yet to be established.

1.8 Calcium homoeostasis

Since Ca^{++} is able to trigger many different cellular events in many different cells, the accurate control of internal calcium concentration represents a fundamental property of all living cells (reviewed by Carafoli, 1987). In excitable tissues and cells of secretory function in particular, the resting intracellular Ca^{++} is tightly controlled at a very low level (10^{-7}M) by a variety of mechanisms, and may increase about 1,000 fold during an action potential (Dipolo & Beange, 1983; Meldolesi & Pozzan, 1987). Persistent, large elevations of intracellular Ca^{++} could have deleterious effects and ultimately lead to cell death. Fig. 1.5 is a schematic view of how a low Ca level could be maintained in a cell. The

calcium enters the cell through various calcium channels in the plasma membrane which may be gated by voltage, hormone receptors or second messengers (reviewed by Meldolesi & Pozzan, 1987; Hardie, 1991). This influx is countered by active transport through the calcium sensitive, calmodulin containing Ca-ATPase and the Na/Ca exchange. Calcium can also be stored in intracellular stores such as the mitochondria and the endoplasmic reticulum or the microsomal stores. There are also calcium binding molecules like calmodulin and synaptophysins which may all have different roles and in turn be regulated through different mechanisms.

1.9 Substances that affect calcium influx

a, Inorganic ions

A number of inorganic ions has been found to reduce pre-synaptic calcium influx and the result of such intervention could block the release of transmitter (Silinskey, 1985). Yamashita et al. (1986) found that Co^{++} and Mg^{++} reduced the responses of the frog type I mechanoreceptor. The problem of using these ions for blocking Ca channels is that they may block (decrease the permeability of) ion channels through the screening effect of surface charges. The screening effect is a common property of divalent ions, including Ca^{++} itself, and affects more then just the voltage gated Ca channels (D'Arrigo, 1973; Green & Andersen, 1991). In fact, the stability of the cell membrane is very sensitive to extracellular calcium concentration due to this reason. In many systems, it may be difficult to distinguish the screening effect from blocking of a specific ion channel.

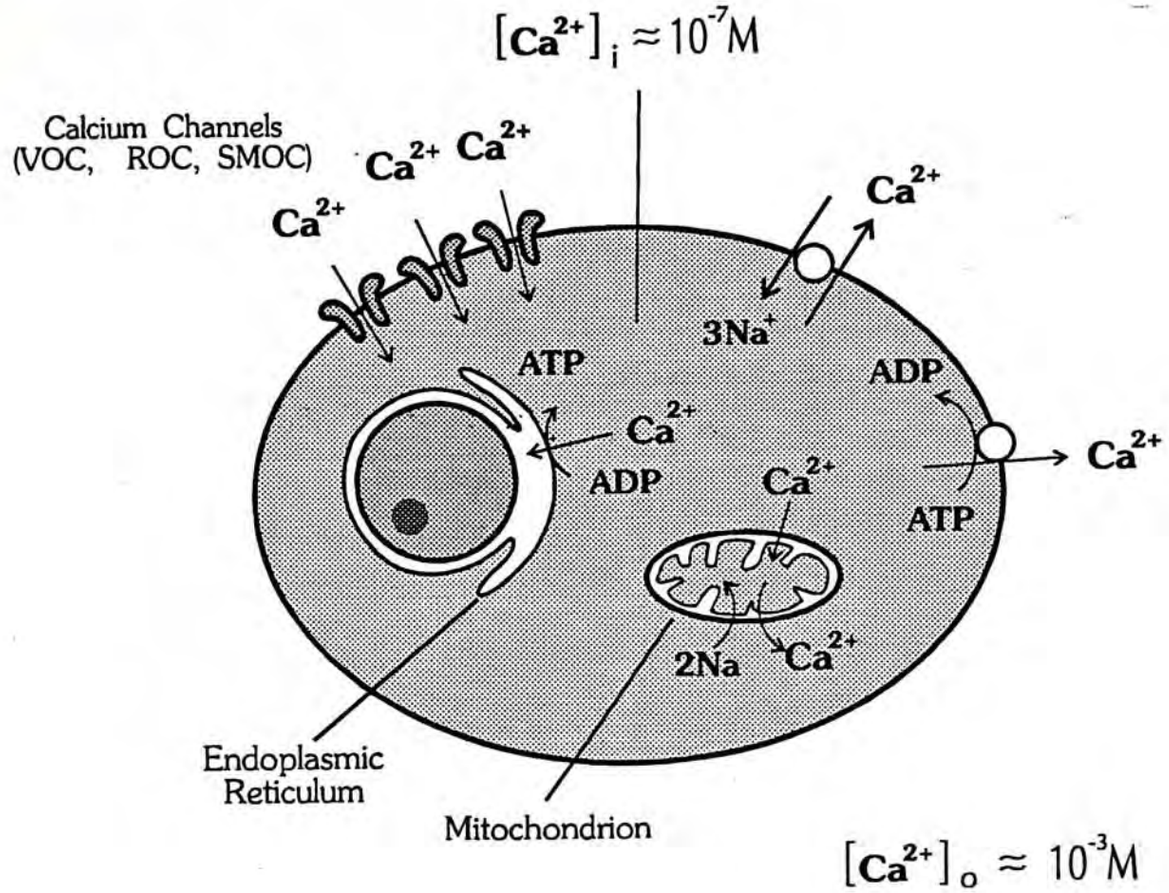


Fig 1.5 Schematic diagram of the various Calcium regulation mechanisms in a cell. Modified and redrawn after Hardie (1991).

b, Neomycin

Neomycin is one of the aminoglycoside antibiotics which are known to impair the function of hair cells in the inner ear (Hudspeth & Kroese, 1983). The mechanism of the action of neomycin is thought to be related to calcium. Neomycin/calcium antagonism has been shown in many tissues including skeletal muscle (Tsai, 1987), central synapses (Fiekers, 1983) and clonal GH3 pituitary cells (Skukla & Wakade, 1991; Suarez-Kurtz & Reuben, 1987). Neomycin has also been shown to decrease the mechano-electrical current in hair cells of the inner ear (Hudspeth & Kroese, 1983) where Ca^{++} has been found to be a major component of the mechano-tranduction current (Ohmori, 1984 and 1985). Previous studies in this laboratory (Baumann et al., 1990) have demonstrated that neomycin reversibly depressed the response of SA I receptors in rats and cats.

c, Verapamil

Verapamil belongs to a group of organic compounds, the dihydropyridines, which bind to calcium channels with very high affinity and have very potent effects on Ca currents in a number of tissues (Reuter, 1983; Smith & Augustine, 1988). Verapamil was found to depress the response of SA I receptors in the rat (Pacitti & Findlater, 1988) and this has been taken to support the notion that Ca influx and transmitter release are important in the function of the SA I receptor. But high concentrations of verapamil can have non-specific effects and could interfere with the function of sodium channels and impair axonal conduction (Chang et al., 1988 and 1989).

d, Bay K 8644

Bay K 8644, an analogue of the 1,4-dihydropyridine calcium channel blockers, has been reported to promote Ca^{++} influx through voltage dependent Ca channels in various tissues (Prues et al., 1985; Williams & Ward, 1991; Brown et al., 1984). Bay K 8644 has also been found to facilitate Ca dependent transmitter release (Albus et al., 1984). Single channel studies have revealed that Bay K 8644 shifted the voltage dependence of L-type channel activation towards more negative potentials and increased the channel opening time (Fox et al., 1987; Markwardt & Nilius, 1988; Ochi et al., 1984). Since the influx of extracellular Ca is believed to be an essential step in pre-synaptic transmitter release, Bay K 8644 is expected to have a stimulatory effect on the SA I receptors if transmitter release is involved in the process. However, in addition this substance may also directly affect a transducer current carried by Ca^{++} ions. There have also been reports of actions of Bay K 8644 at the mitochondrial site, increasing the uptake of calcium into the mitochondria (Baydoun et al., 1990).

1.10 Modulators of intracellular calcium stores.

Interfering with trans-membrane calcium influx has been shown to have potent effects on transmitter release (Augustine et al., 1985). Calcium is thought to be the trigger that couples membrane events to intracellular events responsible for the release of transmitters. Calcium released intracellularly by caged compounds without the involvement of membrane channels could achieve the same (Mulkey & Zucker, 1991). In either case, these extra calcium ions in the cytosol are rapidly removed or relocated to restore the normal intracellular Ca^{++} concentration. This can be achieved in several ways. The Ca pump and Ca/Na exchange in the

plasma membrane can remove calcium from the cell. Alternatively, calcium ions can be relocated into intracellular stores such as the mitochondria and the endoplasmic reticulum, or can be bound to calcium binding molecules in the cytosol. Therefore, in addition to the influx of calcium through calcium channels in the plasma membrane, the release of calcium ions from these intracellular stores or the blocking of uptake of calcium by these intracellular stores can also lead to a rise in intracellular calcium concentration. In the present study, this provided an additional tool to alter the availability of calcium ions to the Merkel Cell.

1.11 Caffeine and cAMP

Studies of the actions of caffeine on translocation of intracellular calcium have been carried out mostly on skeletal muscle. Caffeine augments the twitch response of isolated muscles which is thought to be the result of sensitisation of the mechanism for the release of Ca^{++} from the sarcoplasmic reticulum (see Bianchi, 1975). The same mechanism was also observed in cells with secretory function, such as for the release of catecholamines from the adrenal medulla. Theophylline (1mM) which is a similar substance, could induce secretion in the absence of extracellular Ca^{++} (Peach, 1972)

In a number of other tissues caffeine also has a strong stimulatory effect through the blocking of phosphodiesterase which breaks down cAMP. The resultant increase in cAMP triggers a number of different responses in different tissues. In skeletal muscle, the cAMP pathway is not thought to be involved in caffeine-induced spontaneous contractures (Howel et al., 1981; Kumbaraci & Nastuk, 1982).

Cyclic AMP however, affects a variety of processes including calcium currents in many tissues. In hepatocytes for example, it has been demonstrated that a cAMP-dependent mechanism caused a marked increase in frequency and peak values of free Ca^{++} concentration of α_1-induced calcium transients (Schofl et al., 1991). The cAMP second messenger system has also been demonstrated to be responsible for the mediation of hyperalgesia in mammalian peripheral primary afferent nociceptors (Taiwo et al., 1989).

In the present study, the role of cAMP was tested with membrane permeable analogues like 8-Bromo cAMP, Dibutryl-cAMP and CPT-cAMP which are able to accumulate in cells through diffusion and trigger cAMP dependent mechanisms (Schofl et al., 1991; Eckert et al., 1990; Taiwo et al., 1989) or indirectly with the Caffeine analogue isobutylmethylxanthine (IBMX) which is a very potent phosphodiesterase inhibitor.

1.12 The Mitochondria and NaN3

Mitochondria are abundant in various pre-synaptic terminals in the central nervous system. They are also found abundantly near vesicles in sensory cells, such as the chemoreceptors in the carotid body (Biscoe et al., 1989) and hair cells in the acoustico-lateral system (Flock, 1965). Mitochondria have a large calcium buffering capacity and are thought to play an important role in calcium homoeostasis and the mechanism of transmitter release (Alneas & Rahamimoff, 1975; Carafoli, 1987). Ca^{++} is electrochemically driven into the mitochondria by the membrane potential of the inner mitochondrial membrane ($\approx$160mV, positive outside) maintained by the electron transport chain which also drives the synthesis

of ATP. However, equilibrium is not reached because the uptake is balanced by electroneutral efflux pathways such as the $Ca^{++}/2Na^{+}$ antiport. The kinetics of the mitochondrial uptake and efflux system are such that at cytosolic Ca^{++} concentrations above $\approx 1\mu M$, the efflux pathway is saturated, and the mitochondria will take up Ca^{++} until the cytosolic concentration is under $\approx 1\mu M$. The mitochondria therefore buffer the cytosolic Ca^{++} concentration at around $1\mu M$ (reviewed by Hardie, 1991). Disruption of the mitochondrial electron transport chain by poisons like CN^{-}, NaN_3 or Ruthenium red would disrupt the mitochondrial membrane potential and lead to a rise in intracellular calcium. This can result in a large increase in transmitter release demonstrated in the neuro-muscular junction by Anwyl & Lee (1983) and sensory neurons by Duchen et al. (1990).

SECTION 2. METHODS

2.1 The Nerve-Skin In-Vitro Preparation

Sprague-Dawley rats of about 300 g body weight were anaesthetised with urethane (20% w/v: 6ml/kg I.P.). The saphenous nerve and a large part of the skin of the hind leg innervated by that nerve were carefully dissected free from the underlying muscles in the subcutaneous tissue layer. The skin flap was mounted upside down in an organ bath and superfused with 5ml/min oxygenated artificial tissue fluid at 28°C (Fig. 2.1). The bottom of the bath was lined with a layer of silicone rubber (Silgard, Dow Corning). Stainless steel insect pins were used to fix the skin onto the Silgard bottom. Care was taken to allow a moderate tension in the skin imitating the natural situation. The middle part of the Silgard bottom was made softer than the outer parts so that during mechanical stimulation, the skin was pressed against a softer cushion. The harder outer part of the Silgard was needed to hold the insect pins firmly in place. This was an adaptation of the method described originally by Reeh (1986).

The nerve was then carefully guided through a hole in one side of the perfusion chamber into the recording chamber which was filled with liquid paraffin. Fine filaments were dissected from the nerve and placed on the recording electrodes. Single units were identified and located by probing the skin with a blunt glass rod for their characteristic responses.

2.2 Synthetic Interstitial fluid.

The Synthetic Interstitial Fluid (SIF) described by Bretag (1969) was used in this preparation. The main feature of the composition of the SIF

as compared to Krebs or Tyrode solution is its low (1.5mM) calcium concentration (Table 2.1). The calcium concentration in Krebs or Tyrode was estimated from plasma where a major portion of the calcium is bound to proteins and other molecules. In contrast, SIF approximates only the free calcium in interstitial fluid. The only organic cation gluconate used in SIF is not calcium binding. The SIF was oxygenated with 95% O_2 and 5% CO_2 in a 1.5m high column.

2.3 Localised superfusion

When a suitable single unit was found, a stainless steel ring of 8 mm inner diameter was then placed on the skin surrounding the receptor forming a tight seal. Solutions of a different chemical composition could then be circulated inside the ring via a double barrelled catheter (Fig. 2.2a). This allowed to expose only the receptor site and distal end of the afferent nerve fibre to the test solutions. During control periods before and after the test solution, the inside of ring was perfused with the normal artificial tissue fluid as the rest of the bath.

2.4 Nerve chamber perfusion

On some occasions, the ring was placed not on the receptor site but right next to it on the nerve leading to the receptor being stimulated. This allowed to distinguish between drug effects on the receptor mechanism and those on nerve conduction. For later experiments an additional perfusion chamber for the nerve was attached to the septum that separated the main chamber and the recording chamber (Fig. 2.2b). When this was in place, the nerve was guided through this chamber before entering the recording chamber.

	S.I.F. (Bretag, 1969)	Krebs (1950)	Tyrode (1910)	Pacitti & Findlater (1988)
Na	145	143	149	116
K	3.5	5.9	2.7	5.4
Ca	1.5	2.54	1.8	2.5
Mg	0.69	1.18	1.05	1.2
Cl	114	128	145	128
HCO_3	26.2	24.9	11.9	-
Phosphate	1.7	1.18	0.42	-
SO_4	0.69	1.18	-	-
Gluconate	9.6	-	-	-
Glucose	5.55	11.1	5.55	-
Sucrose	7.6	-	-	-
Dextran	-	-	-	5.6
Hepes	-	-	-	10

Table 2.1 Comparison of the Compositions of Several Bathing Solutions. (All concentrations in mM)

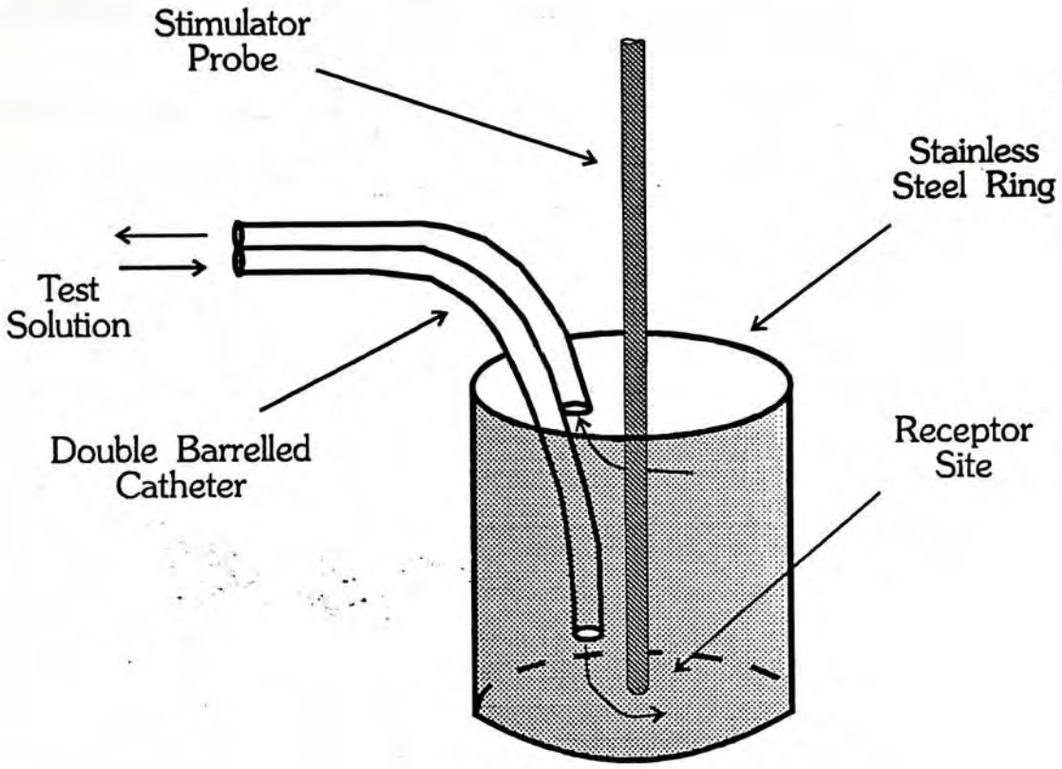


Fig 2.2a Localized perfusion of the receptor site is achieved with a stainless steel ring and a double barrelled catheter.

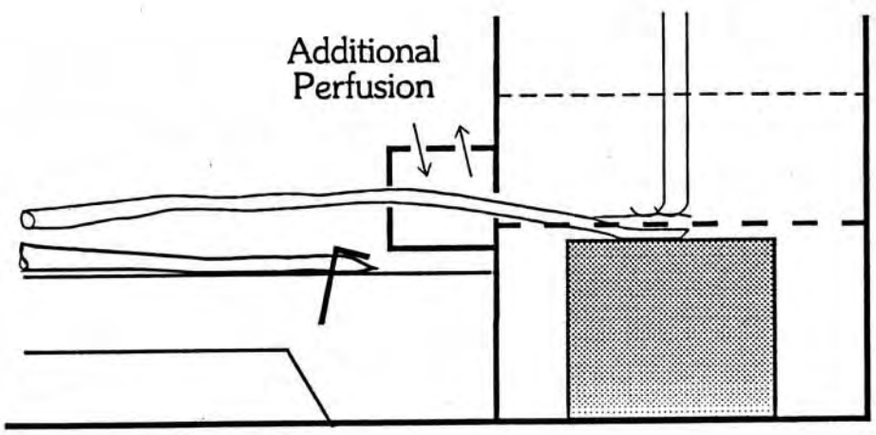


Fig 2.2b Perfusion of the nerve is done in an additional chamber attached to the wall of the recording chamber.

2.5 Mechanical stimulation and experiment control (Fig. 2.3)

Stimulus control, data capture, on line and off line analyses were done mainly through a 80386 based microcomputer linked with the CED1401 Lab interface. Mechanical stimuli were controlled by a feedback unit developed in conjunction with the CUHK Electronics Department. Constant force mechanical stimuli were applied every 30 seconds to the receptor site through a Perspex rod with a spherical tip. Each stimulus rose from a contact force of 0.5 mN within 200 ms to a plateau force which was kept constant by the feed back control unit for further 2 seconds. Force, displacement of the probe and action potentials from the nerve including the trigger level of the Schmitt trigger were monitored on an oscilloscope and recorded on a chart recorder. The original signal from the nerve, after being amplified and band filtered, along with the signal from the force transducer were also digitized by a modified sound digitizer (Sony PCM 601) and recorded on video tapes. The discriminated spikes and timing signals were fed into the CED1401 Lab interface for on line analysis and stored on files. In particular, the timing of each action potential was sequentially filed on the hard disk of the computer. This allowed the reconstruction of the post-stimulus-time profile from which inter-spike-interval (ISI) histograms of any part of the stimulus cycle could be readily derived for each and every stimulus.

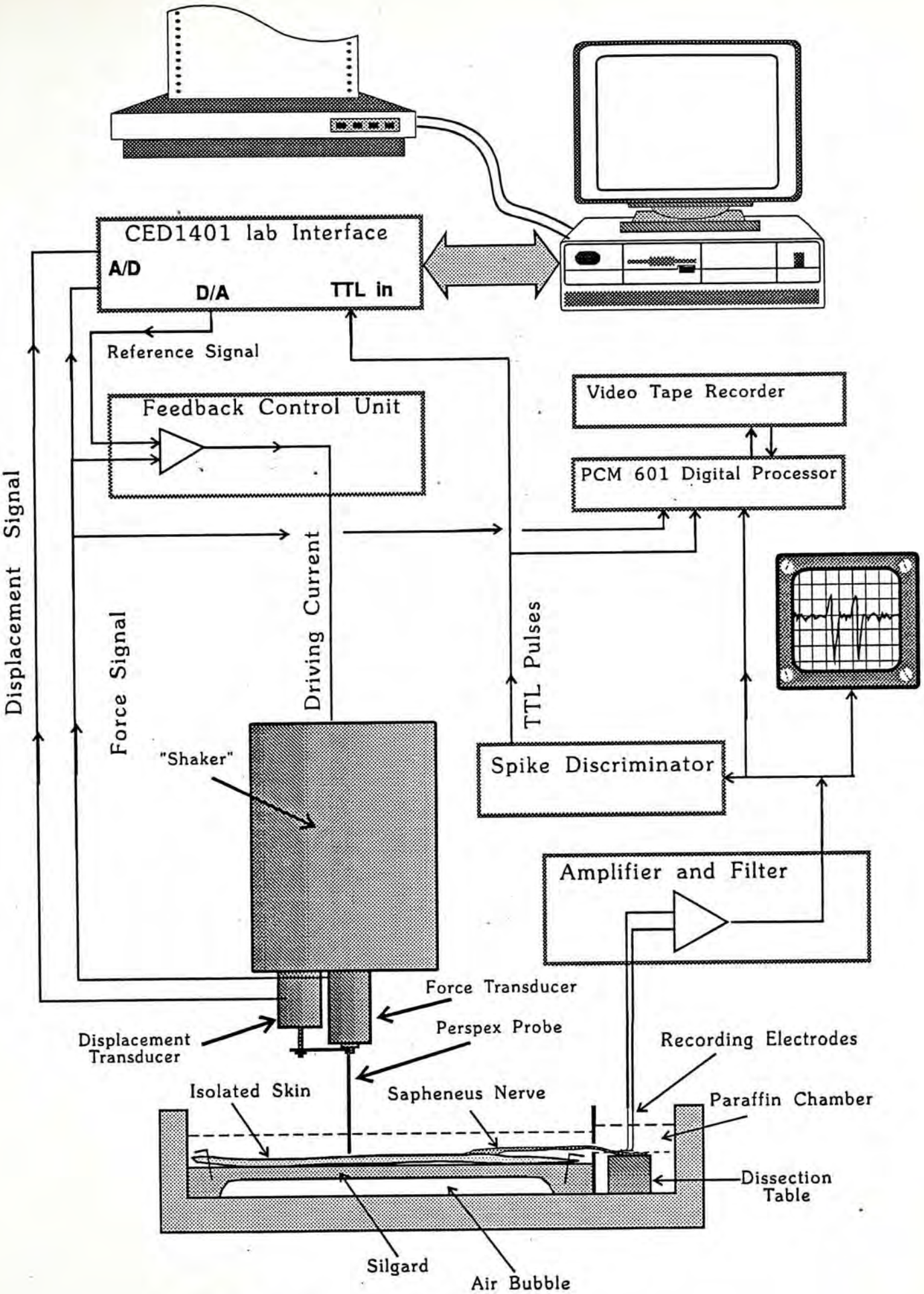


Fig. 2.3 Block diagram of the experimental setup.

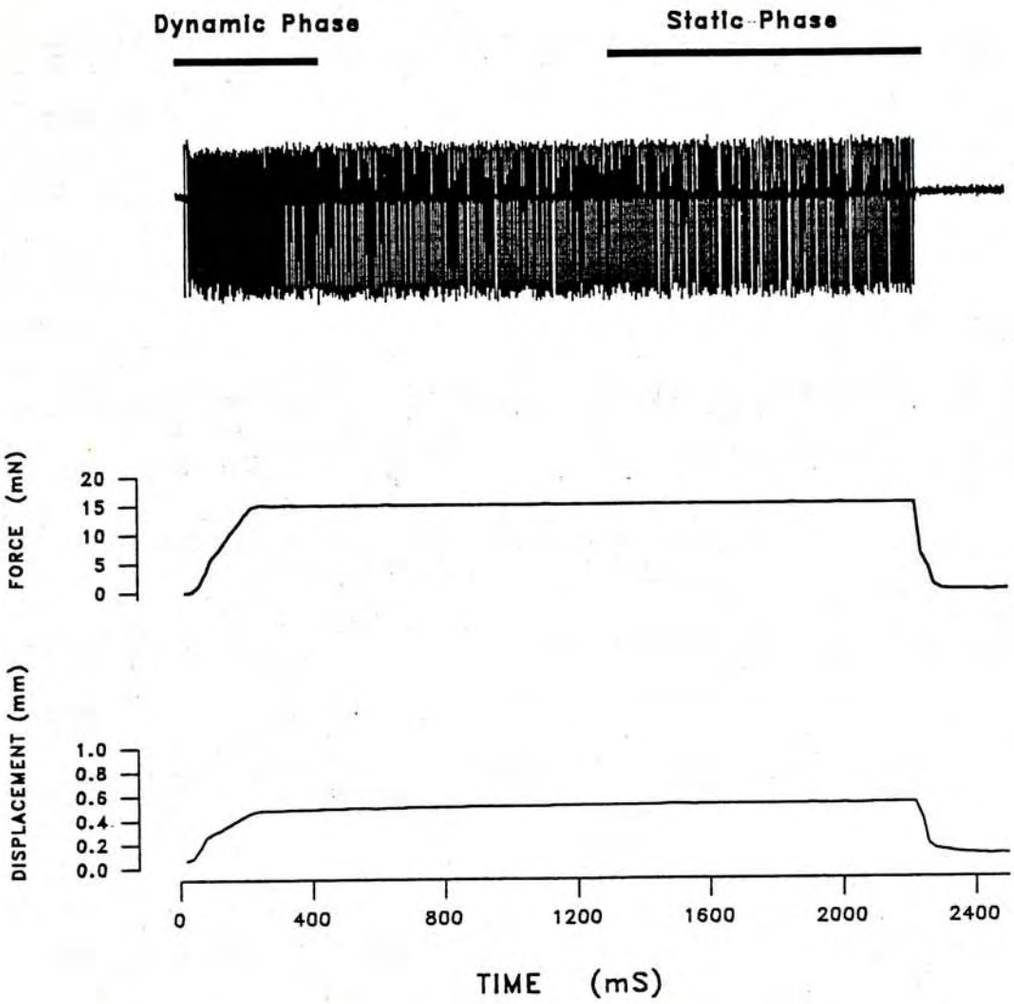


Fig. 2.4 Typical response of a SAI receptor to a standard mechanical stimulus.

2.5 Data Analysis

Fig. 2.4 shows a typical response of a SA I receptor to a standard mechanical stimulus. Receptor responses were usually expressed as total number of impulses per stimulus. The firing rate was highest during the dynamic phase of the mechanical stimulus when the force rose from 0.5 mN contact force to the 15 mN pre-set plateau force, and adapted to lower discharge rates during the plateau phase of stimulation. Receptor responses are expressed as total number of nerve impulses per stimulus and subdivided into dynamic response (during the first 300 ms) and static response (last 1000 ms of the plateau phase).

Receptor responses are also expressed in relative terms where the average response of the last 10 minutes before the application of a drug was taken as the control period, and responses to individual stimuli were then expressed as percentage of this control level.

For each action potential the time since the onset of the mechanical stimulus was recorded in a post-stimulus time file using a bin width of 1 ms and used to compute the Inter-spike-interval histograms for the dynamic and static phase of the stimuli. These were also displayed on the computer monitor immediately after each stimulus. This further assisted the proper identification of SA I and SA II receptors.

SECTION 3. RESULTS

3.1 Viability of the preparation

Long Term Stability

The in vitro skin-nerve preparation was found to be well suited for experiments on cutaneous mechano-receptors lasting for several hours. Fig. 3.1.1a shows responses of a SA I receptor to standard mechanical stimulation every 30 s over a period of five hours. There was a slow decline in the responses over this period of time of about 5 % per hour. The typical shape of the inter-spike-interval (ISI) histograms was maintained during that time (Fig. 3.1.1b).

Force response

The relationship between the applied force and the responses of SA I receptors was tested and the results of two units are shown in Fig. 3.1.2. In these experiment the plateau force was varied between 5 mN and 25 mN in random sequence. The resulting receptor responses were highly reproducible. The typical shape of ISI histograms could be observed at all stimulation forces. With increasing number of impulses per stimulus the distribution became condensed at short intervals. For most experiments a plateau force of 15 mN was employed. Discharge rates in response to this force were high enough for reliable analysis of ISI histograms but still submaximal. Thus, drug effects increasing or decreasing responses could be observed equally well.

Firing pattern of type I and type II units

Type I units were much more abundantly found in the isolated skin preparation then type II receptors (Ruffini endings). This is likely to be due to the fact that type II receptors are located in the subcutaneous tissue and may be partly damaged by the dissection process. ISI histograms were obtained from the last 1000 ms of each stimulus when the firing was well adapted. Fig. 3.1.3 compares the typical ISI histograms of type I (SA I) and type II (SA II) mechanoreceptors. The firing rate of this type II receptor was very regular with most of the intervals between spikes falling into the range between 22 and 26 ms (C.V.=0.095). In contrast, the firing of type I receptors was much more irregular, here with a C.V. of 0.45 resembling a Gamma distribution. It was a general observation in SA I receptors that the coefficient of variance (C.V.) increased when the discharge rate decreased (see figs. 3.1.1 and 3.1.2). In contrast, the C.V. for the discharge of type II receptors tended to decrease with lower discharge rates (see fig. 3.2.5 on page 44).

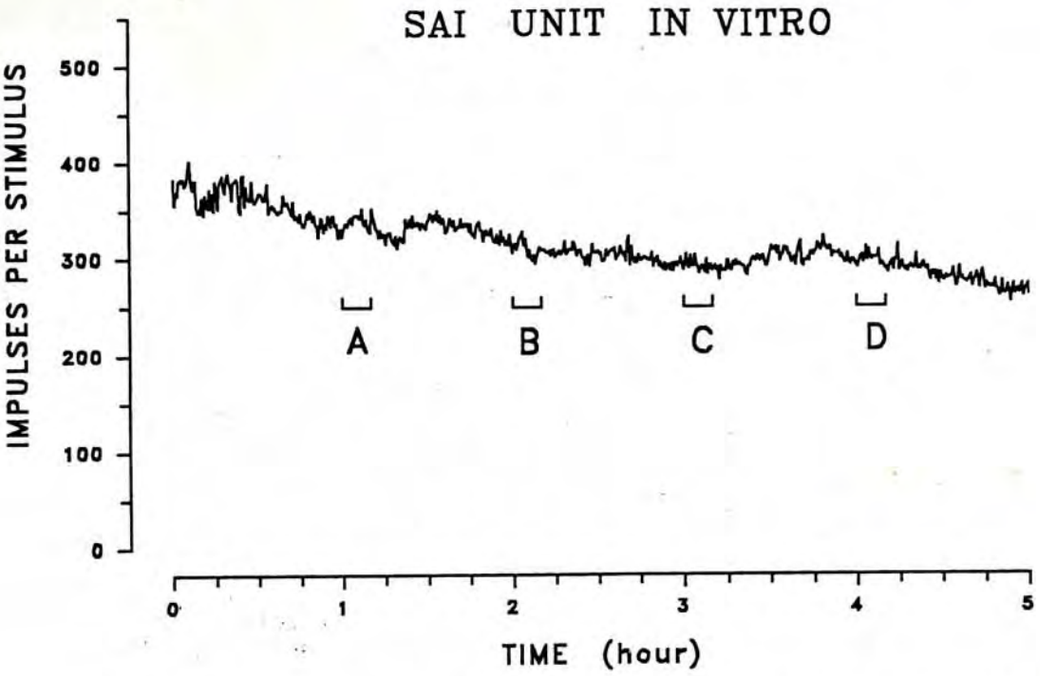


Figure 3.1.1a Response of a normal SAI unit showing stability over five hours.

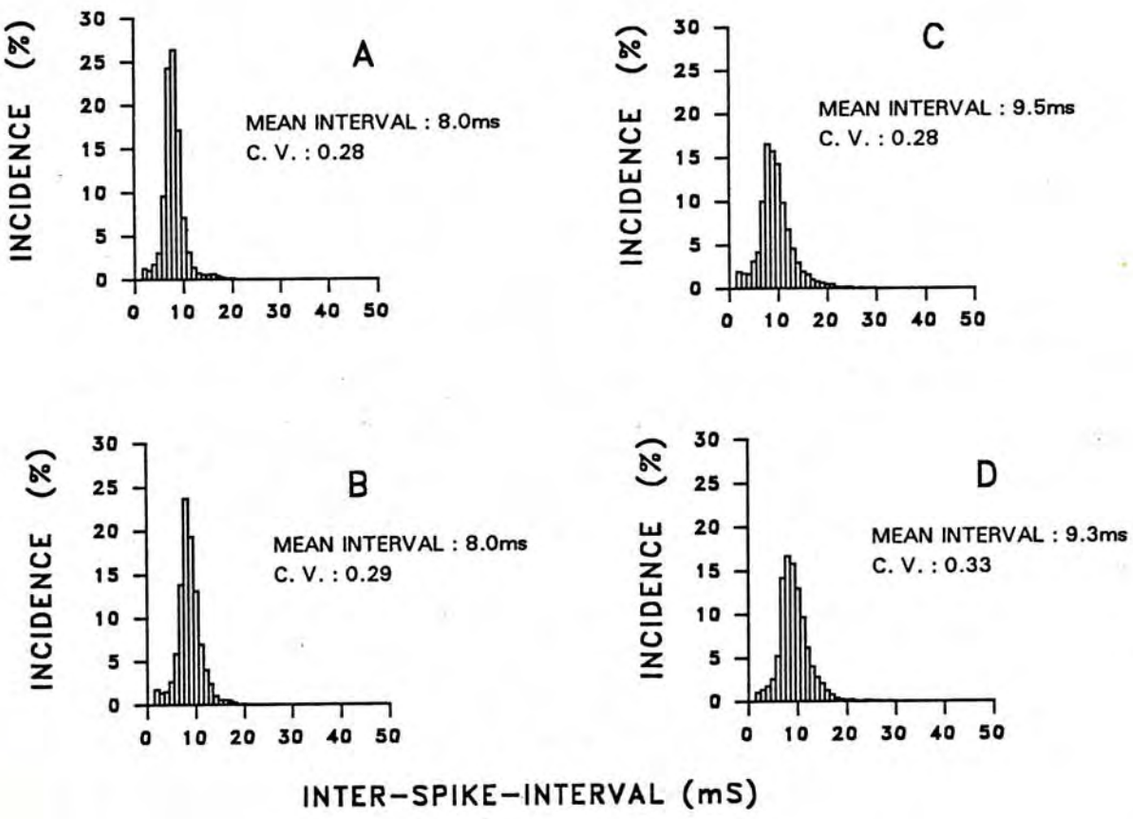


Figure 3.1.1b ISI histogram of the same unit showing the firing pattern well maintained throughout the entire five hours.

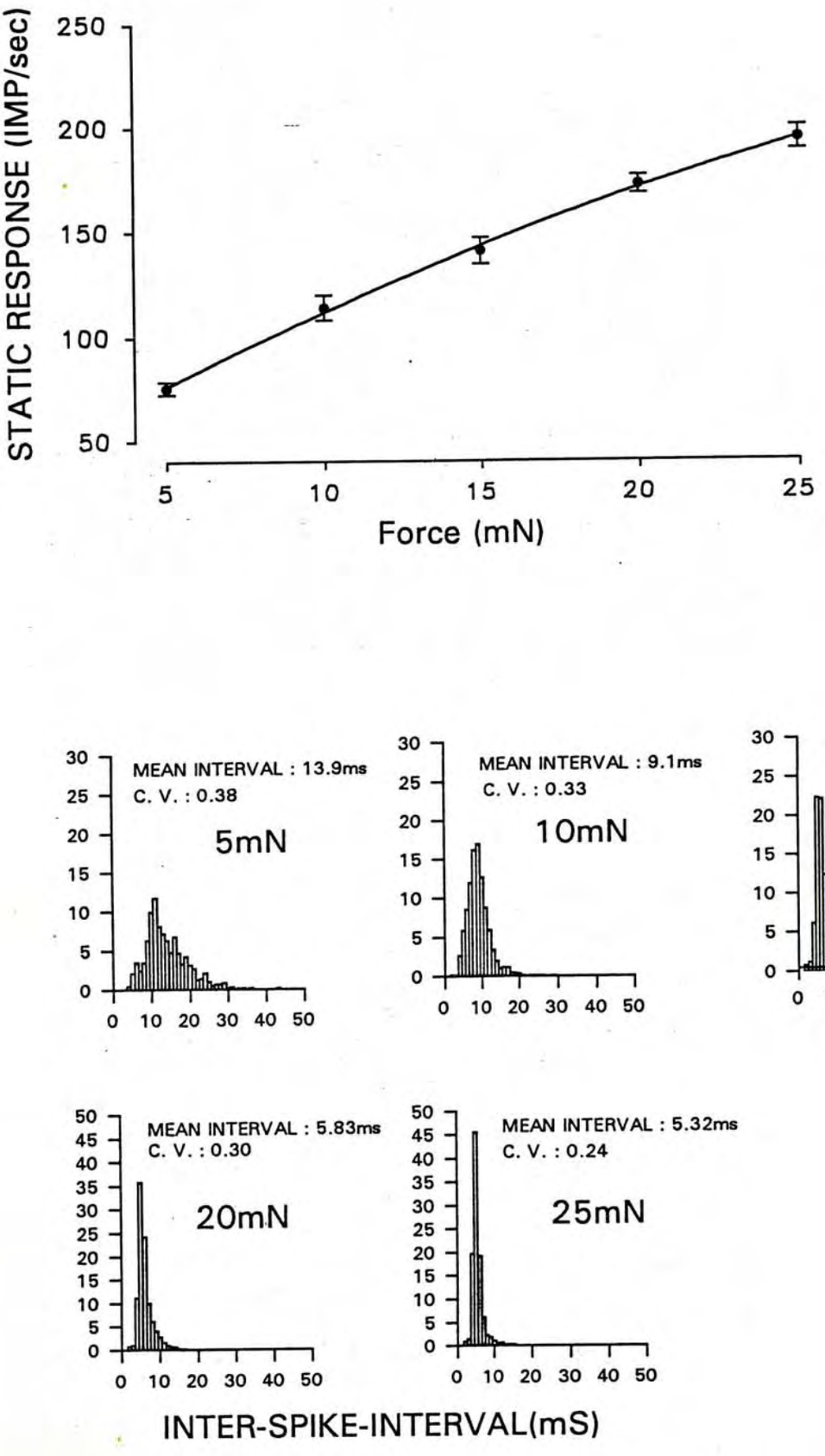


Fig. 3.1.2 Relation between applied force and receptor responses in SAI receptor.

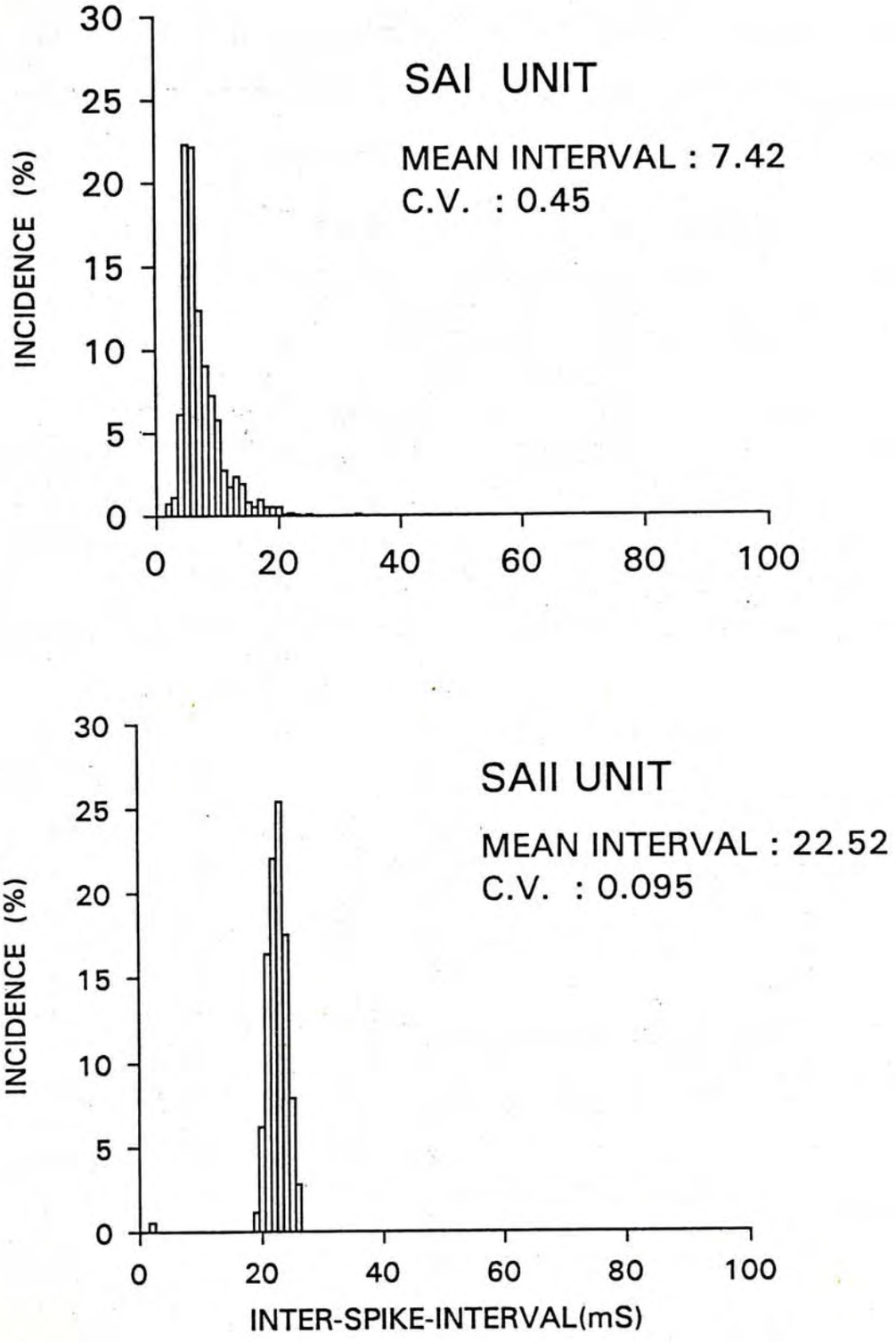


Fig. 3.1.3 Typical responses of SAI and SAII receptors

3.2 Effects of Neomycin

The aminoglycoside antibiotic neomycin had been shown in previous in situ studies in this laboratory to suppress type I receptor responses to a greater extent than those of type II receptors. In those experiments the concentration of neomycin was not well under control but estimated to be about 0.5 to 1 mM. The effect was therefore re-examined on nine SA I and two SA II units as well as exposing only the afferent nerve (two experiments) to clearly defined neomycin concentrations for short periods of time (typically 10 minutes).

Type I receptors

Neomycin, as solution of neomycin sulphate (Mycifradin) was applied at a concentration of 0.5 mM (0.35 mg/ml) for 10 minutes. Receptor responses dropped sharply to about 40 % of control responses. After switching back to superfusion of the receptor with normal solution responses recovered slowly to between 80 and 100 %. On average this recovery process required about 1 hour (Fig. 3.2.1).

When neomycin was applied at a higher concentration of 3.5 mg/ml (5 mM) for 10 minutes the sharp drop in responses was very similar reaching a minimum of about 30 % of the original value. After returning to control superfusion, recovery was again almost complete requiring up to 2 hours (Fig. 3.2.2).

Displaying the ISI profile for each response throughout the course of such an experiment (Fig. 3.2.3a), a band can be seen shifting towards longer intervals of the spectrum while broadening at the same time and returning slowly back after removal of neomycin. In the usual way of presentation (Fig. 3.2.3b) it can be easily seen how the shape of the ISI histograms is

flattened and the range of intervals broadened under the influence of neomycin.

Type II receptors

Type II receptors (Ruffini endings) were exposed to neomycin solutions in the same way. When neomycin was applied at 0.5 mM, the responses dropped about 50 % and recovered within less than 1 hour to the original response level (Fig. 3.2.4). When exposed to 5 mM neomycin for 10 minutes, the responses dropped more than 80 % but recovered well within about 1 hour of normal superfusion (Fig. 3.2.5). While responses were reduced by neomycin, the ISI histograms remained narrow with low C.V. but shifted towards longer intervals. When the ISIs were displayed for each response (Fig. 3.2.6), a narrow band could be seen shifting towards longer intervals and back without the broadening of the band seen regularly in SA I receptors.

Nerve Conduction

When neomycin (5 mM) was applied only to the nerve through perfusion of the nerve chamber while the receptor site was perfused with the normal solution and stimulated with standard mechanical stimuli, there were no changes in the response (Fig. 3.2.7).

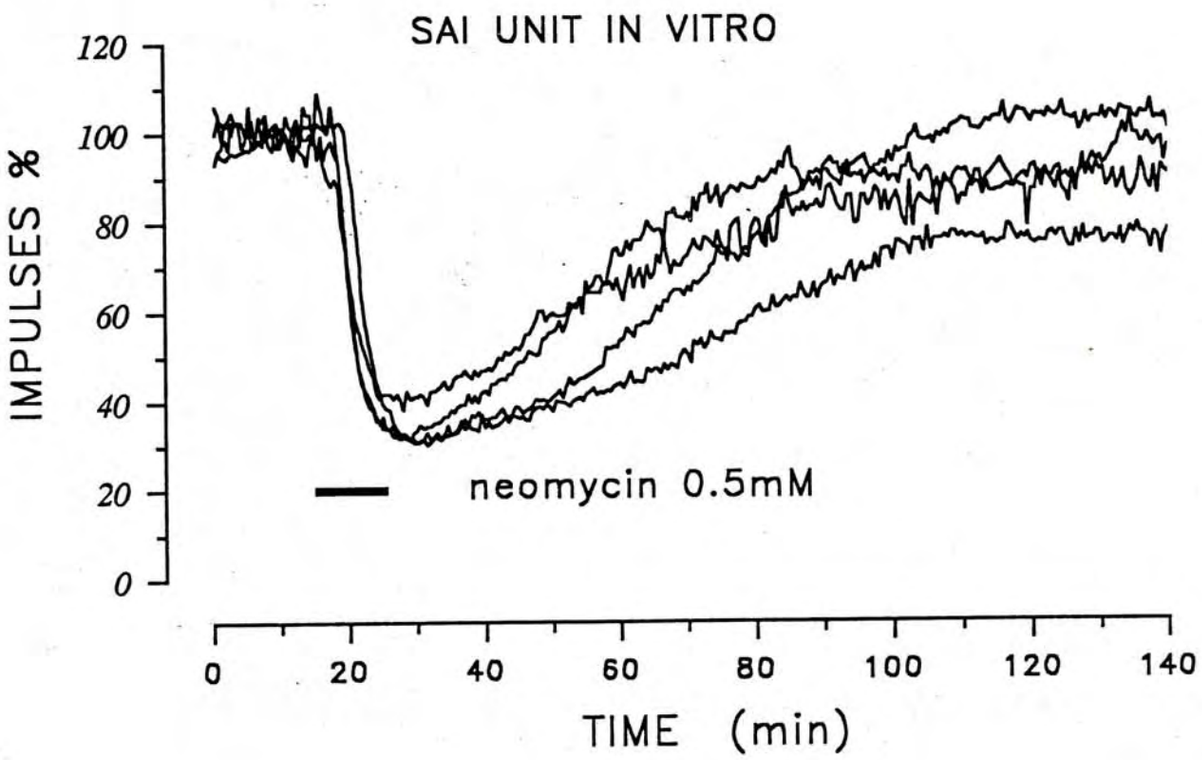


Fig. 3.2.1. Effect of neomycin (0.5mM) on SAI receptors (N=4).

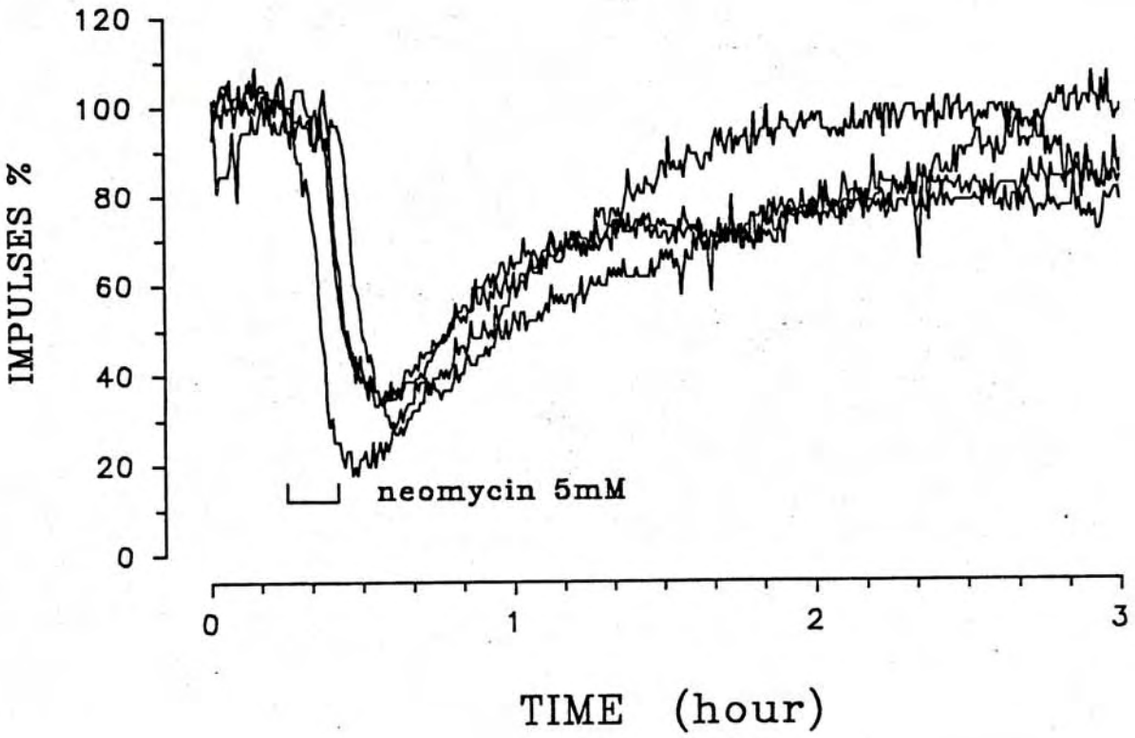


Fig. 3.2.2 Effect of neomycin on SAI receptors.

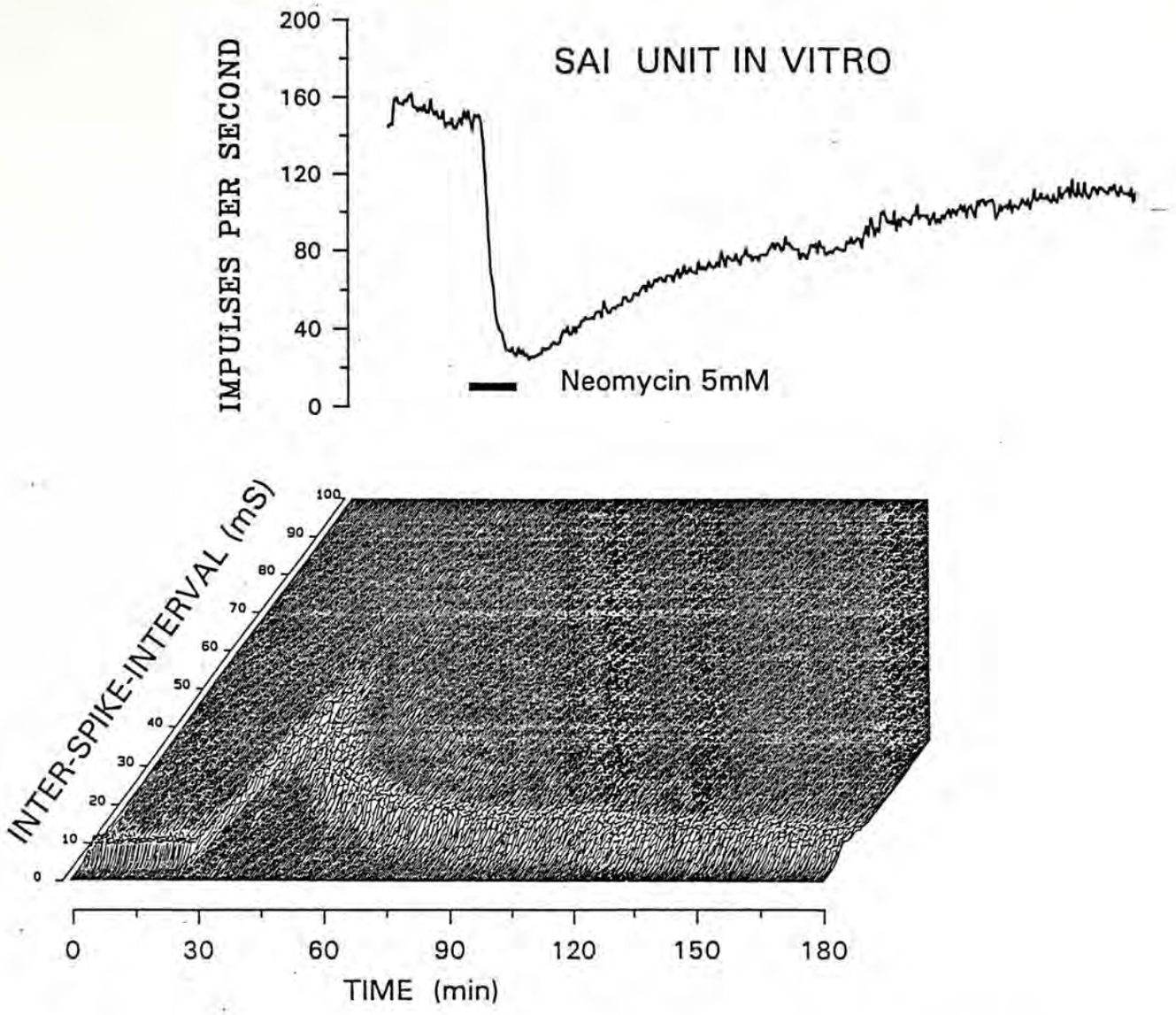


Fig. 3.2.3a Effect of neomycin on a type I receptor. Inter-spike-interval histogram (lower graph) was displayed for each response. Under neomycin the band broadened and shifted towards longer intervals and recovered within two hours after the end of neomycin superfusion.

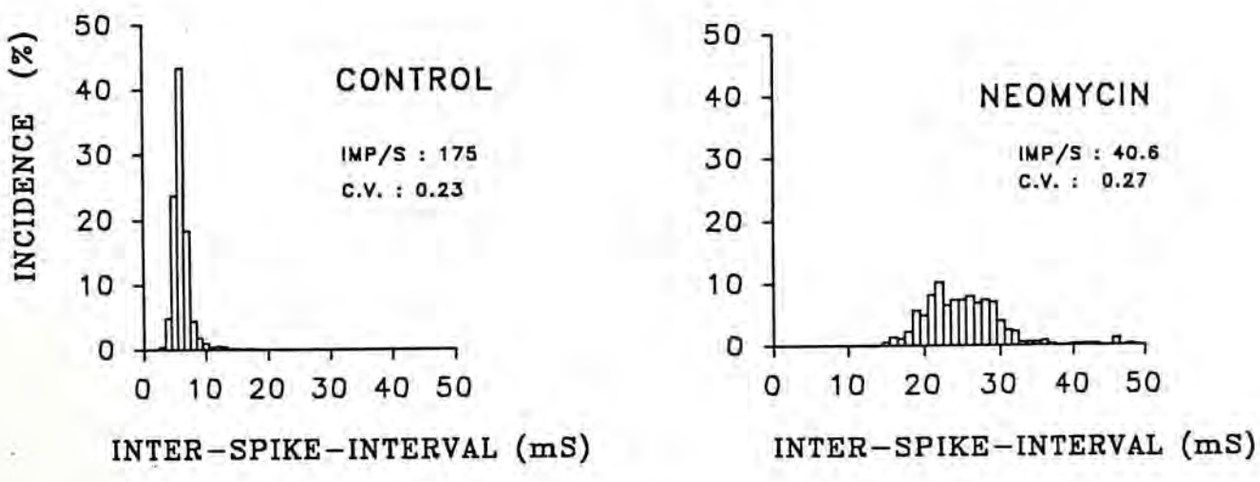


Fig. 3.2.3b ISI histograms during neomycin(right) and during control period(left).

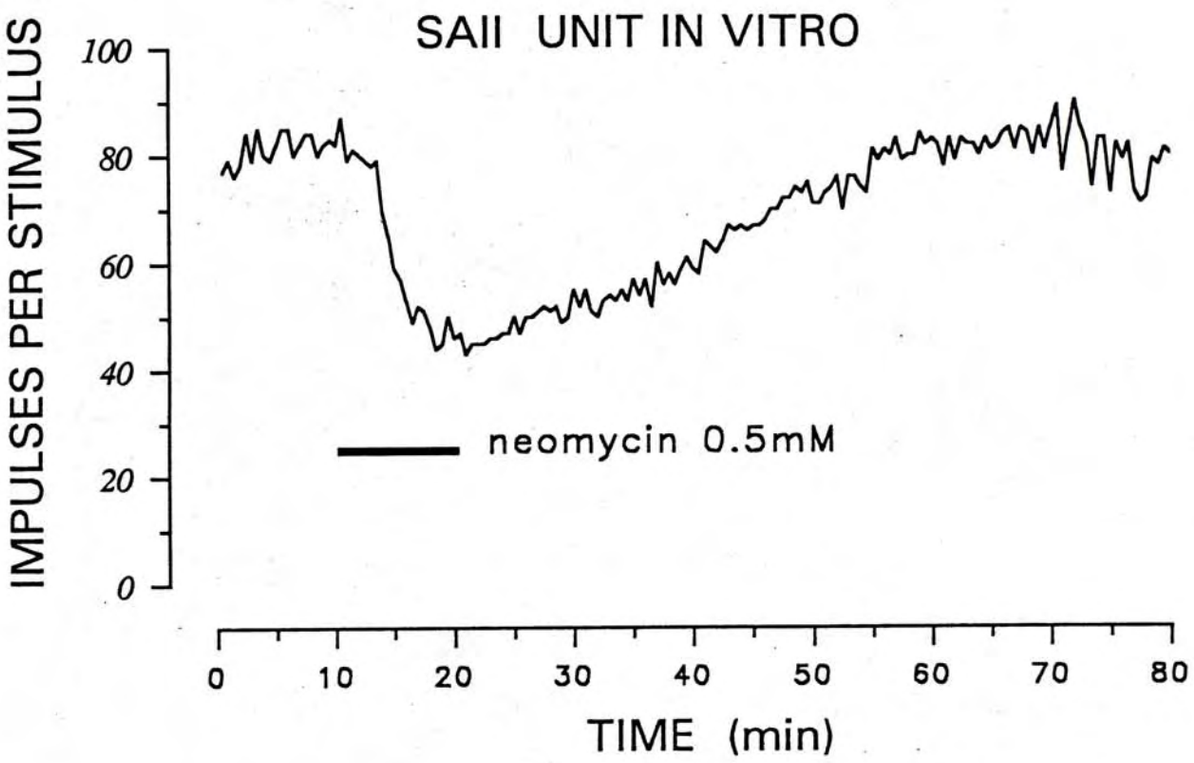


Fig 3.2.4 Effect of Neomycin (0.5mM) on SAII receptor.

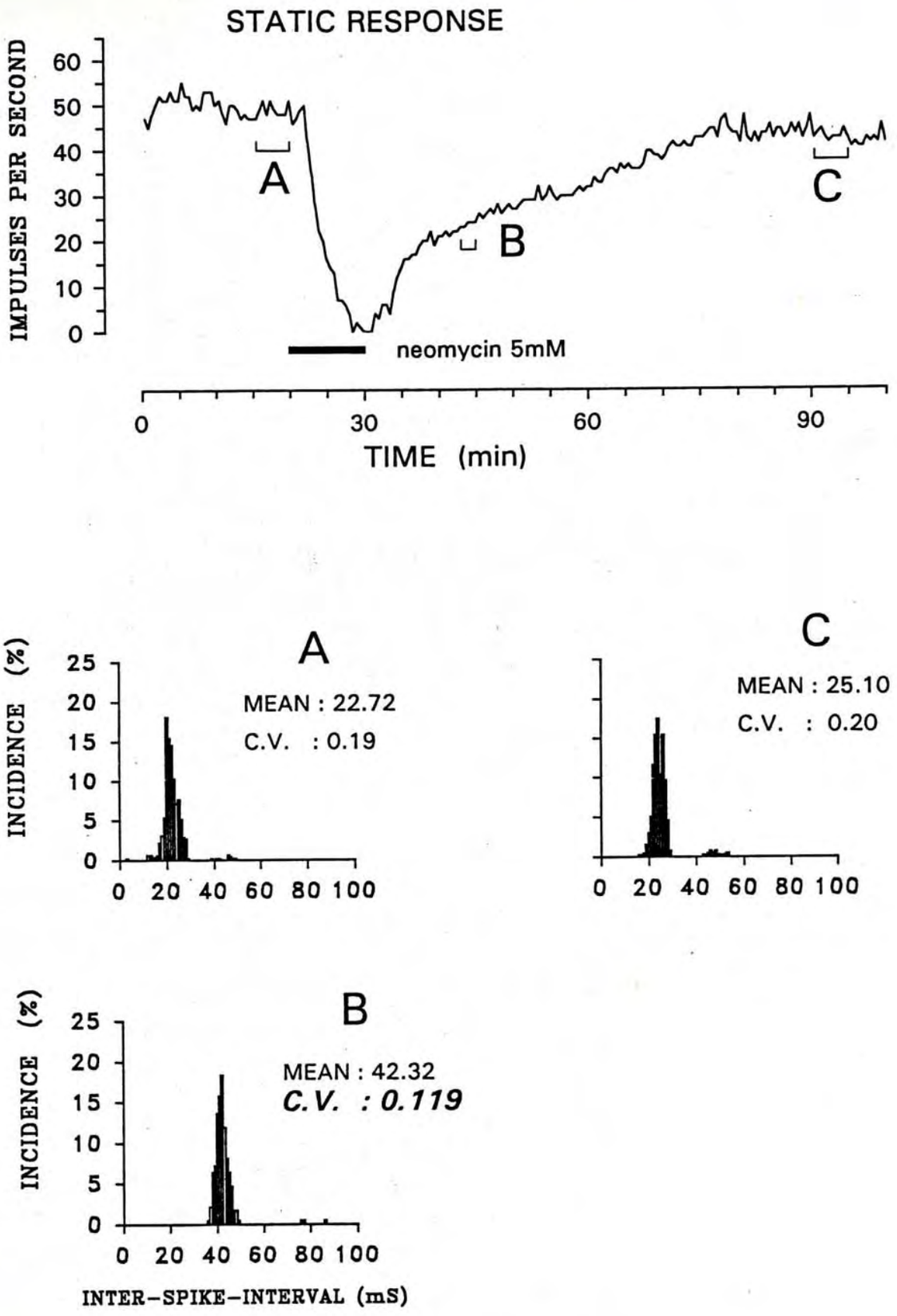


Fig. 3.2.5 Effect of neomycin on type II receptors.

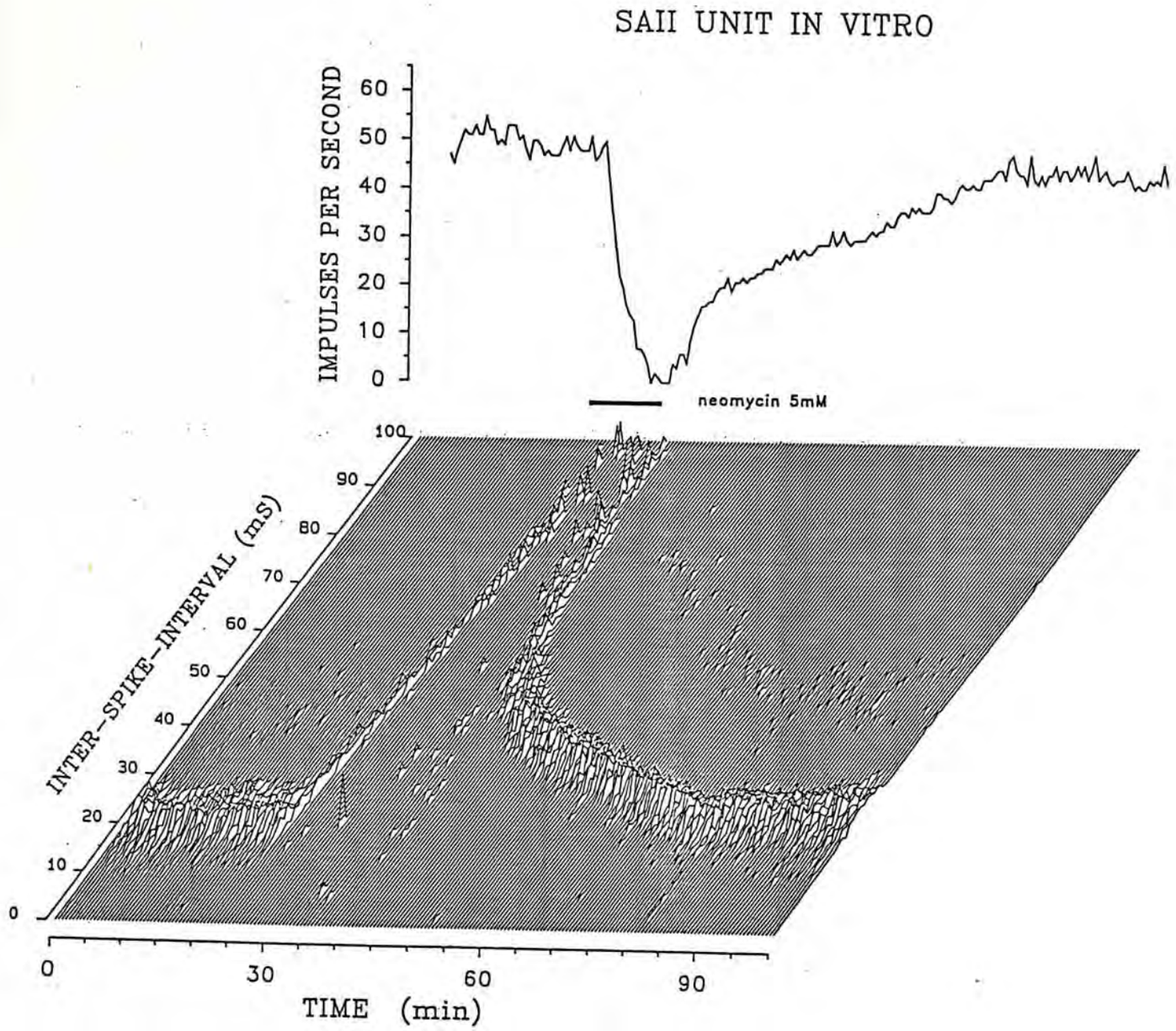


Fig. 3.2.6 Effect of neomycin on type II receptors. Inter-spike-interval histogram (lower graph) was displayed for each response. A band could be seen to shift towards longer intervals and back with little disturbance in shape.

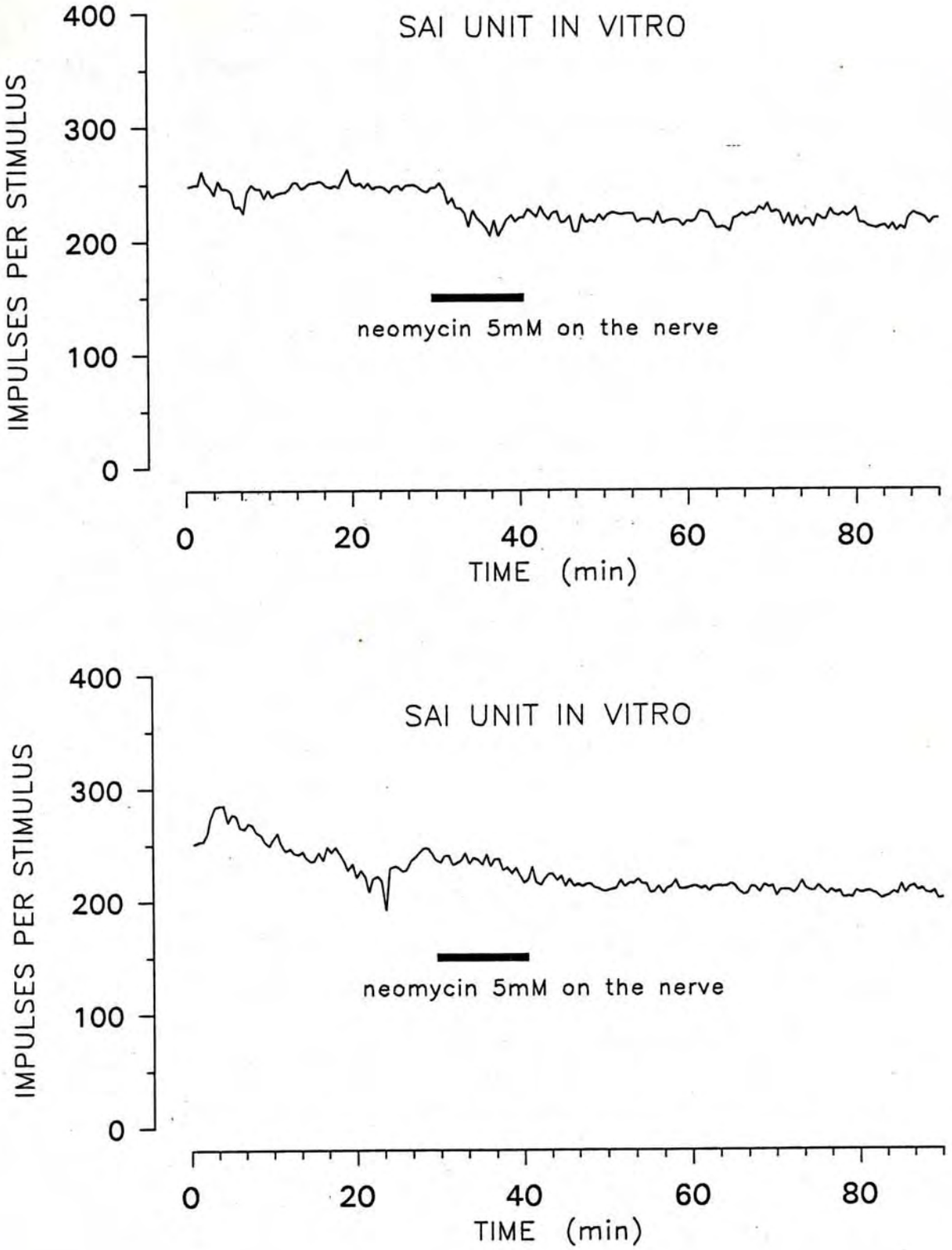


Fig 3.2.7 Effect of neomycin on the nerve was tested in two SAI units, shown here separately for clarity. Receptor responses, shown here as impulses per stimulus, were not affected.

3.3 Effects of Mg^{++}

Mg^{++} is known to competitively block the influx of Ca^{++}-ions through calcium channels. In order to test the role of calcium influx in slowly adapting cutaneous mechanoreceptors ten SA I units and one SA II unit were examined.

Type I receptors

Mg^{++} at concentrations of 5 mM and 10 mM was applied to the receptor sites for 10 minutes. Receptor responses dropped rapidly and recovered quickly upon returning to the normal solution (Fig. 3.3.1). During the time while the responses were suppressed, the ISI histograms were shifted and flattened as a result of the lowered firing rate. The appearance of multiple peaks was not observed under Mg^{++} (Fig. 3.3.2).

Type II receptors

The effects of Mg^{++} on a type II receptor was found to be essentially similar. The total number of impulses in response to mechanical stimulation was lowered by about 20 and 40 % after addition of 5 mM and 10 mM of Mg^{++} respectively (Fig. 3.3.3). During Mg^{++} application, the ISI histograms were shifted towards longer intervals but retained their shape (Fig. 3.3.3). In contrast, the ongoing discharge, recorded as the average discharge rate during the last 10 seconds of the 27.8 s period between stimuli, was found to be affected to a much greater extent than the firing rate in response to 15 mN stimuli (Fig. 3.3.4).

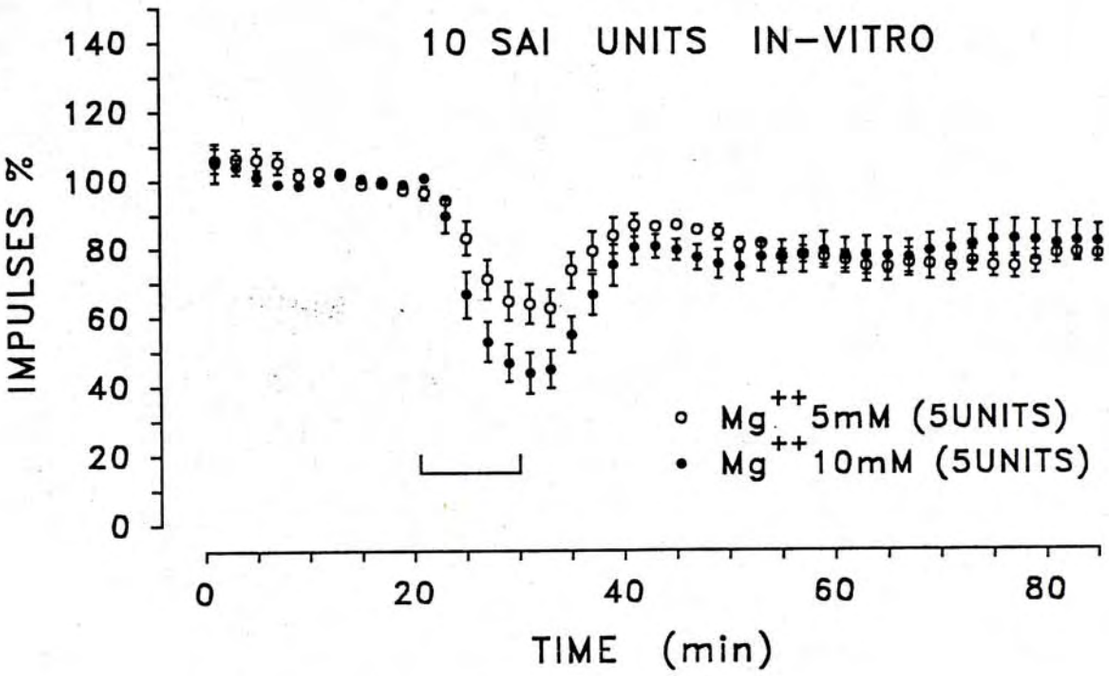


Fig.3.3.1 Effects of Mg^{++}, 10mM and 5mM, on the response of SAI receptors.

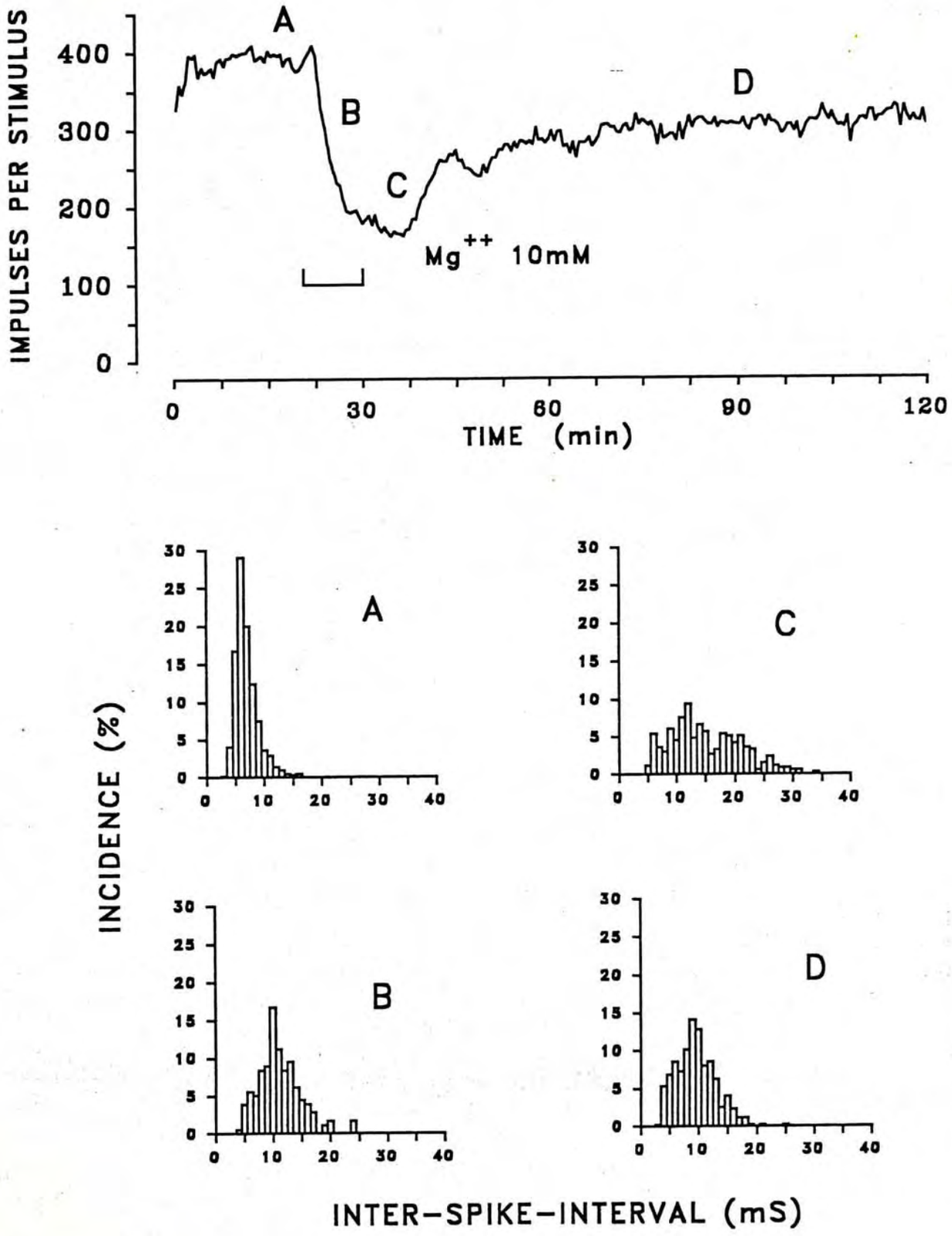


Fig.3.3.2. Response and ISI histograms of a SAI receptor under 10mM Mg^{++}.

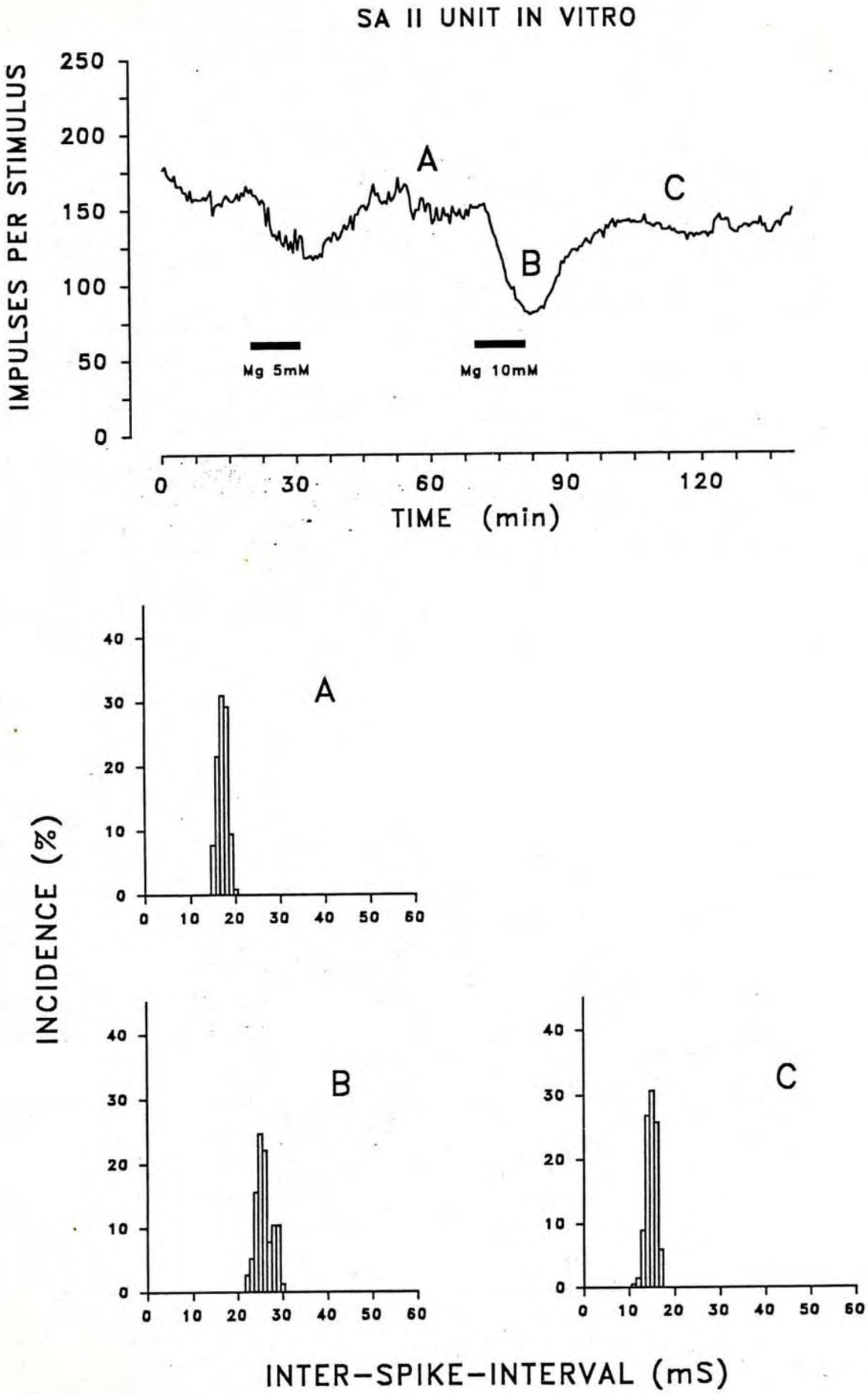


Fig 3.3.3 Effect of Mg^{++}, 5mM and 10mM, on SAII receptor.

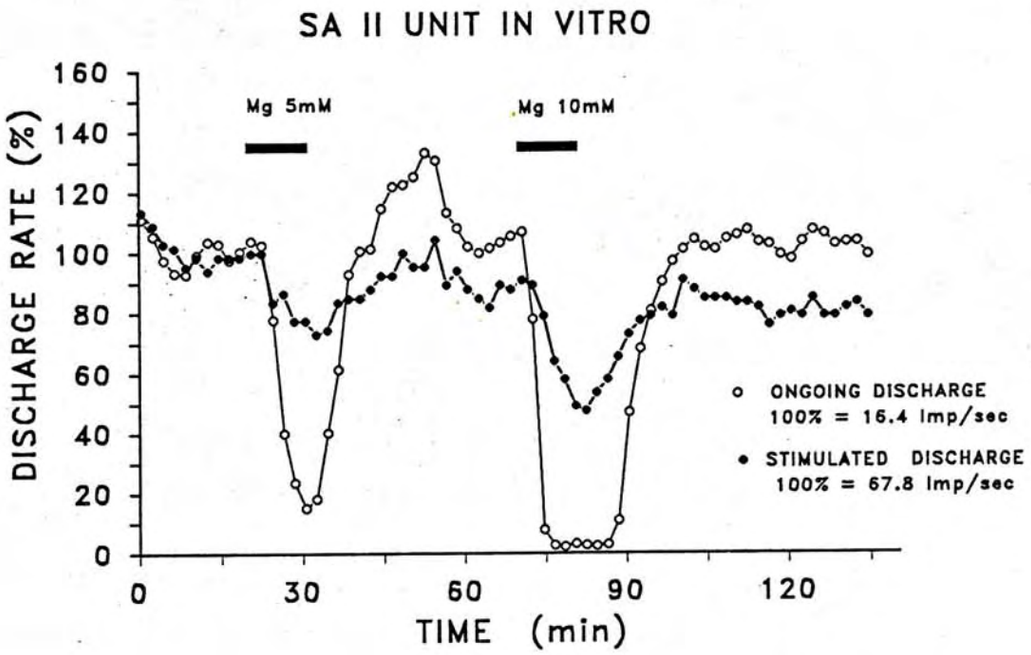


Fig 3.3.4 Effects on stimulated and ongoing discharge of SAII receptor under 5mM and 10mM Mg^{++}

3.4 Effect of Verapamil

Verapamil is a classical blocker for L-type calcium channels and had previously been reported to suppress SA I receptor responses. In the present study ten SA I units and one SA II unit were tested. In addition the effect on nerve conduction was examined by exposing only the afferent nerve to the drug.

Type I receptors

Low doses of verapamil (up to 10 μM) had no significant effect on SA I receptors (Fig. 3.4.1a). Superfusion with 50 μM for 20 minutes usually did not produce a consistent effect. Larger doses of verapamil (100 μM) strongly reduced the number of nerve impulses in response to standard mechanical stimuli in SA I receptors. Thus, it was not easy to establish a dose response relationship.

In all 5 receptors tested, superfusion with 100 μM verapamil produced a very pronounced effect but the time required until the responses dropped very sharply varied between 15 to 25 minutes (Fig. 3.4.1b). After returning to the normal solution, receptor responses started immediately to recover and had usually returned to about the control level within 10 minutes.

During the time while the receptor responses were depressed by verapamil, multiple peaks were consistently observed in the ISI histograms (Fig. 3.4.2). These extra peaks appeared at integer multiples of the original peak. In the experiment shown, the original peak was at 8 ms, smaller peaks can be seen at 16 ms, 24 ms, and 32 ms.

Type II receptors

Only one SA II receptor had been examined under the influence of verapamil due to the scarcity of type II receptors in this preparation (Fig. 3.4.3). The effect on receptor responses was rather similar. The number of impulses dropped to 40 % of the control level when the receptor was exposed to 100 μM of verapamil for 20 minutes. Because SA IIs fire in very regular intervals and normally show a very narrow band in the ISI histograms, the multiple peaks during verapamil superfusion are well separated.

Nerve Conduction

In order to test whether the reduced responses of type I and type II receptors might be caused by impaired conduction of action potentials in the afferent nerve, verapamil (100 μM) was perfused through the nerve chamber exposing a short length (ca.3 mm) to the drug while the receptor was superfused with normal SIF (Fig. 3.4.4). About 15 minutes after the onset of the verapamil perfusion the number of nerve impulses recorded distal from the nerve chamber dropped sharply and again recovered within less than 10 minutes after the perfusion of the nerve chamber was switched back to normal solution.

It should be noted that both during exposure of the receptor or the nerve multiple peaks appeared in the interspike interval histograms. During those periods the first peak remained in the same position as during the control period (Figs. 3.4.2 to 4) suggesting that the original main firing frequency was not affected.

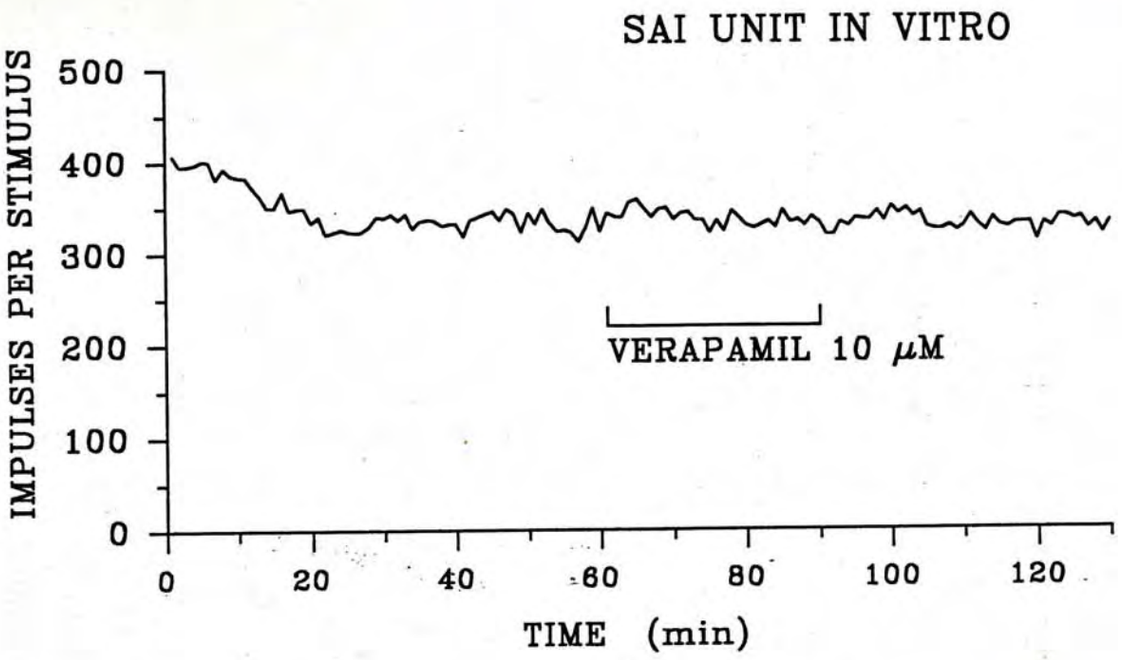


Fig 3.4.1a Effect of Verapamil(10μM) on SAI receptor.

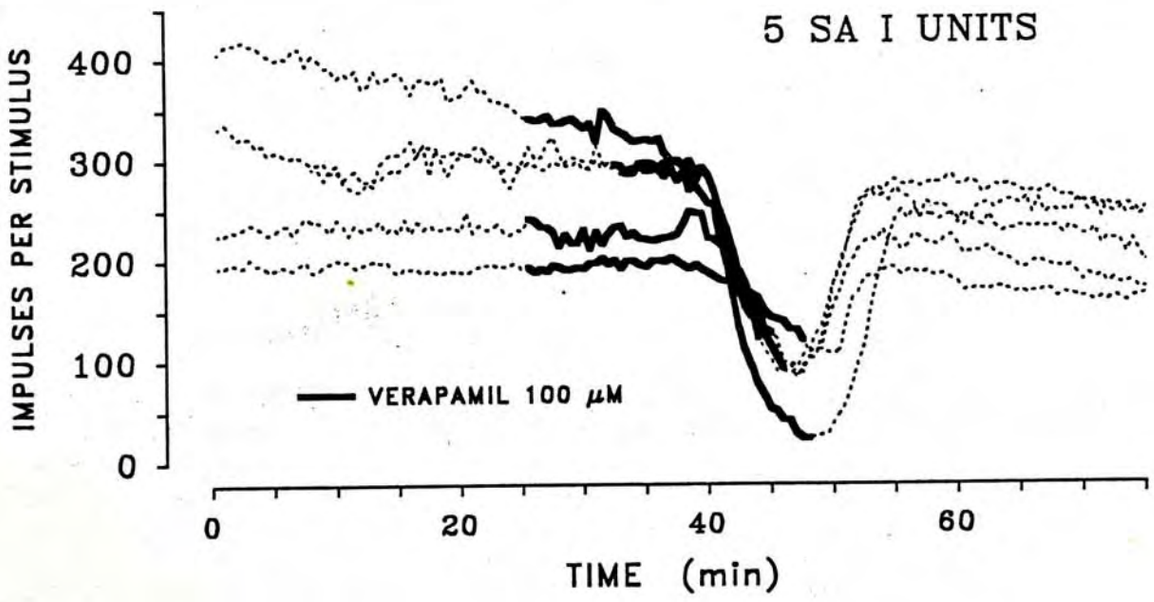


Fig 3.4.1b Effect of Verapamil(100μM) on SAI receptors.

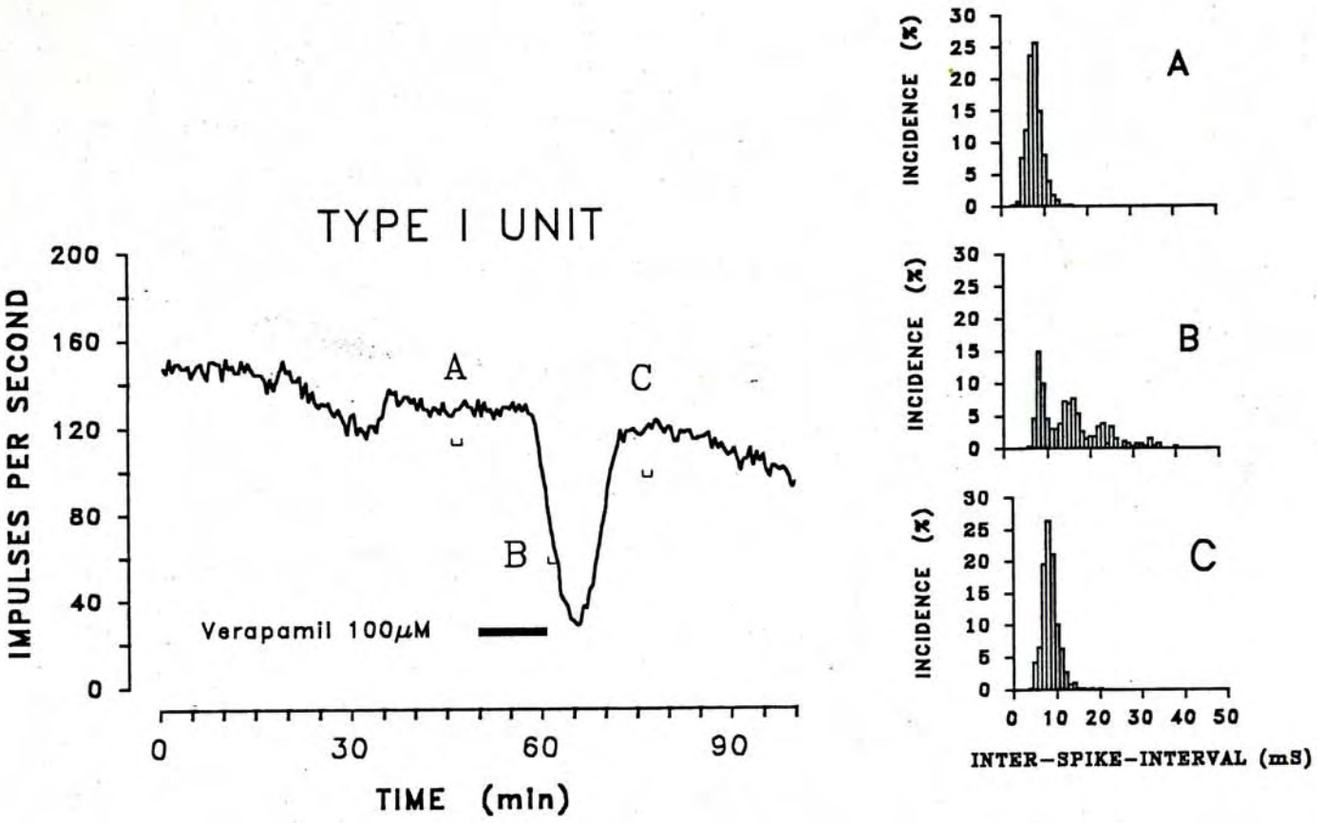


Fig 3.4.2 Response of this SAI receptor was sharply reduced after application of verapamil. The ISI histogram shows multiple peaks at interger multiples of the first peak.

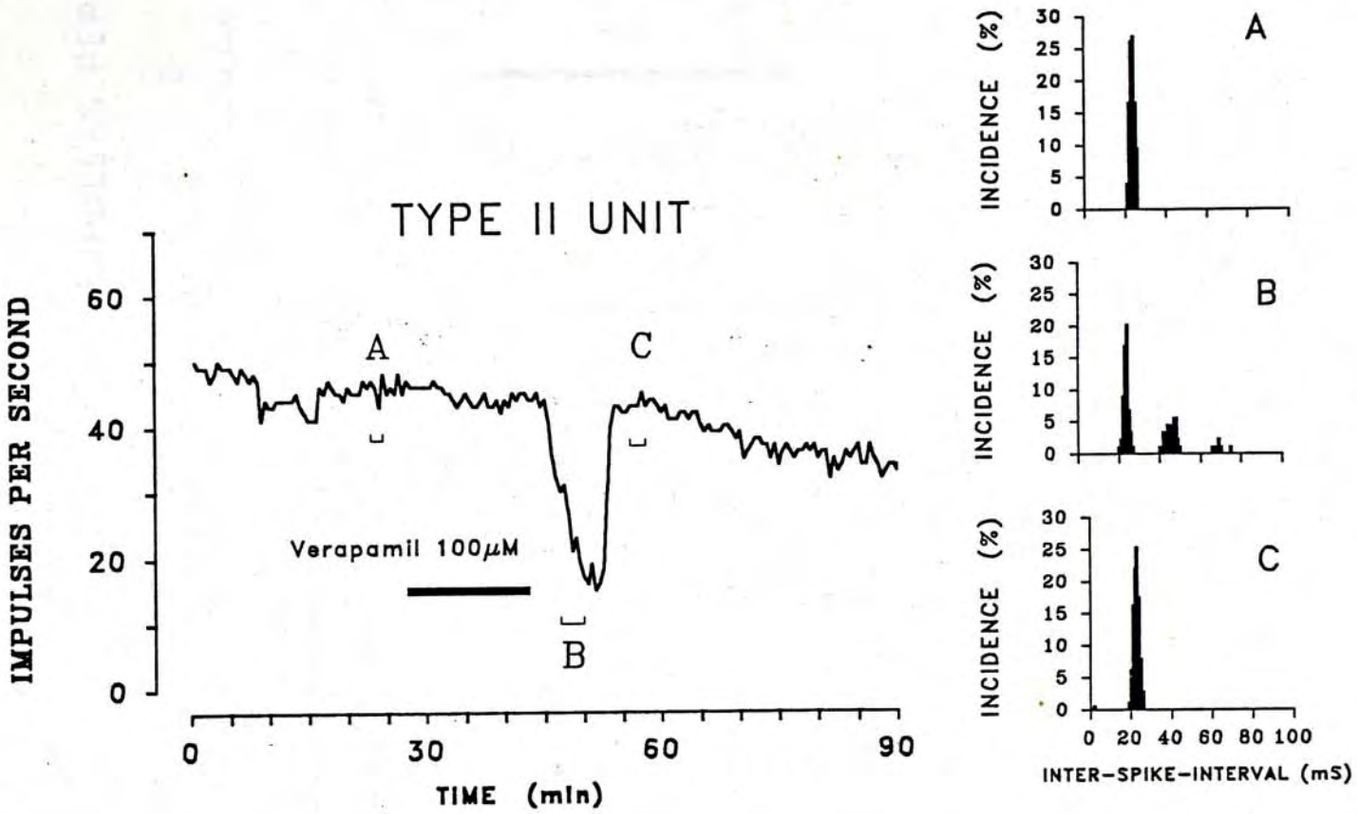


Fig 3.4.3 Response of the SAII receptor was sharply reduced following application of verapamil. During the reduced response, ISI histograms showed multiple peaks at interger multiples of the first peak.

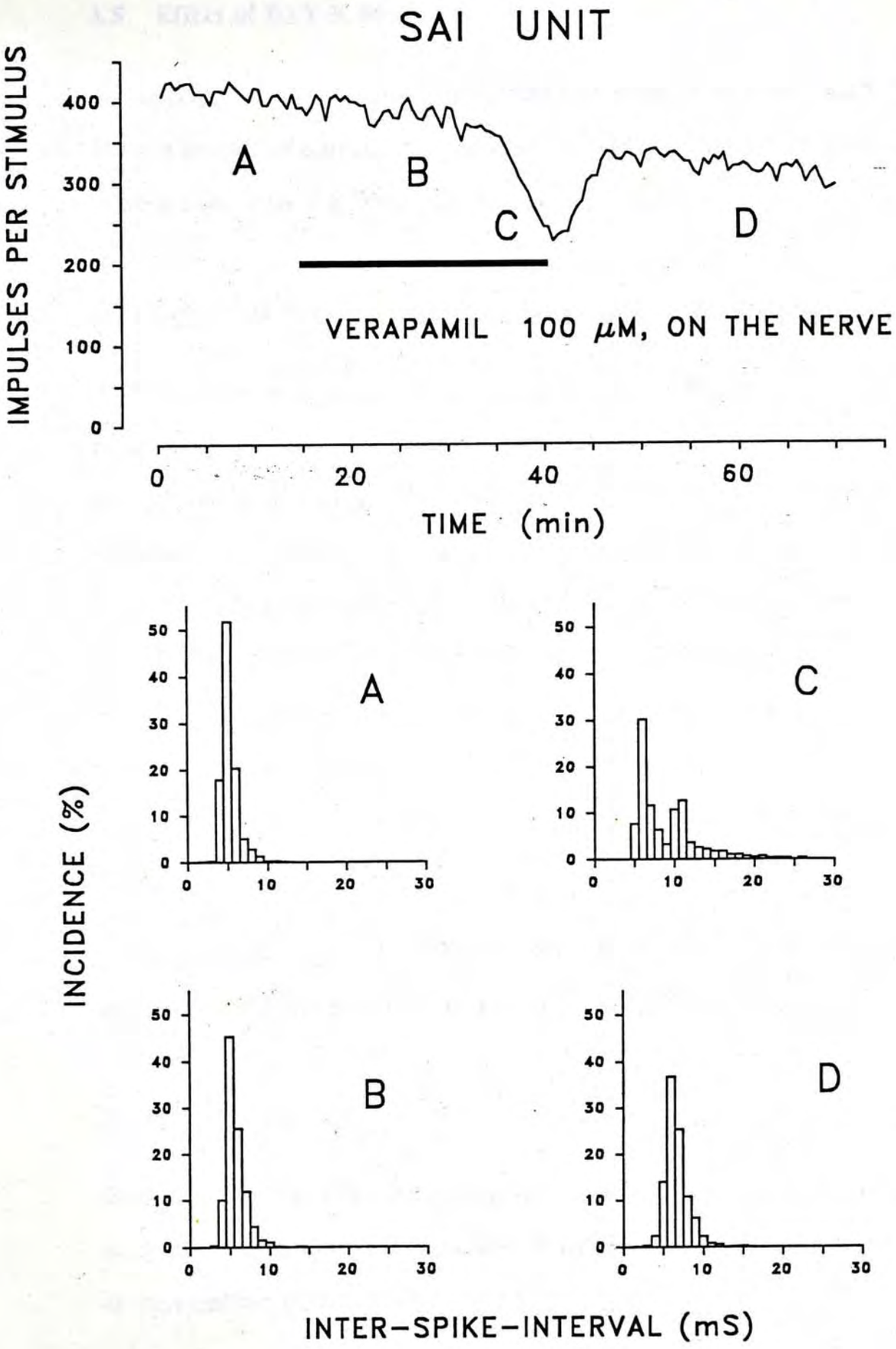


Fig 3.4.4 Effects of Verapamil on the nerve when applied to the nerve chamber. The receptor site was perfused with normal SIF.

3.5 Effect of BAY K 8644

An analogue of the dihydropyridine calcium channel blockers, BAY K 8644 is known to activate calcium channels. BAY K 8644 was tested on five SA I and three SA II units as well as on the nerve.

Type I receptors

BAY K 8644 was found to increase the responsiveness of the SA I receptors. When BAY K 8644 was applied for 15 minutes at concentrations of 10 and 50 μM, the average number of impulses per stimulus was increased by 10 and 25 % respectively (Fig. 3.5.1). Analyzing the static and dynamic components of the total response, the increase was found to affect both phases to a similar degree (Fig. 3.5.2). ISI histograms also revealed that the normal firing pattern was maintained throughout the experiment.

Type II receptors

Similarly applied for 15 min at 50 μM, BAY K 8644 was found to increase the responses of SA II receptors to a similar degree (Fig. 3.5.3).

Nerve Conduction

When BAY K 8644 (50 μM) was applied to the nerve chamber, there was no change in discharge rate or pattern. Thus, BAY K 8644 exerts its effect on the receptors rather than the nerve (Fig. 3.5.3).

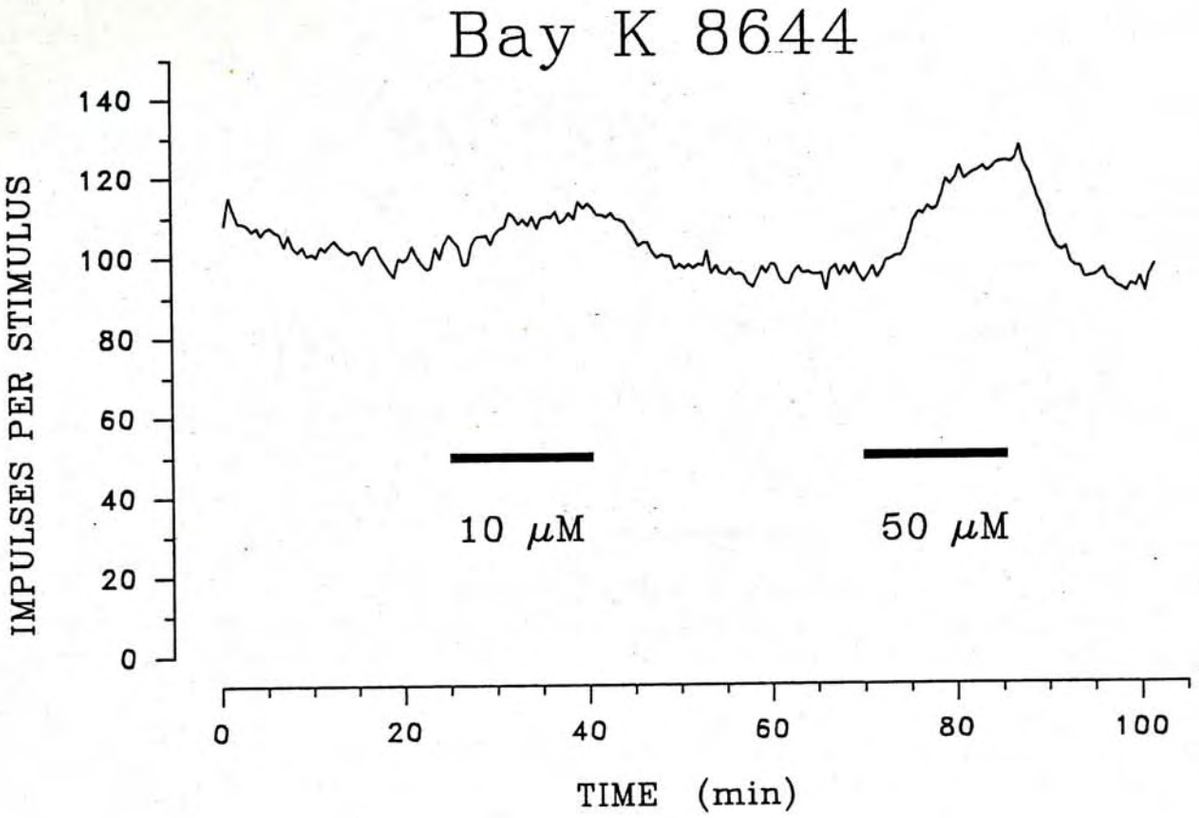


Fig 3.5.1 Responses of a SAI receptor during application of Bay K8644 at 10μM and 50 μM respectively

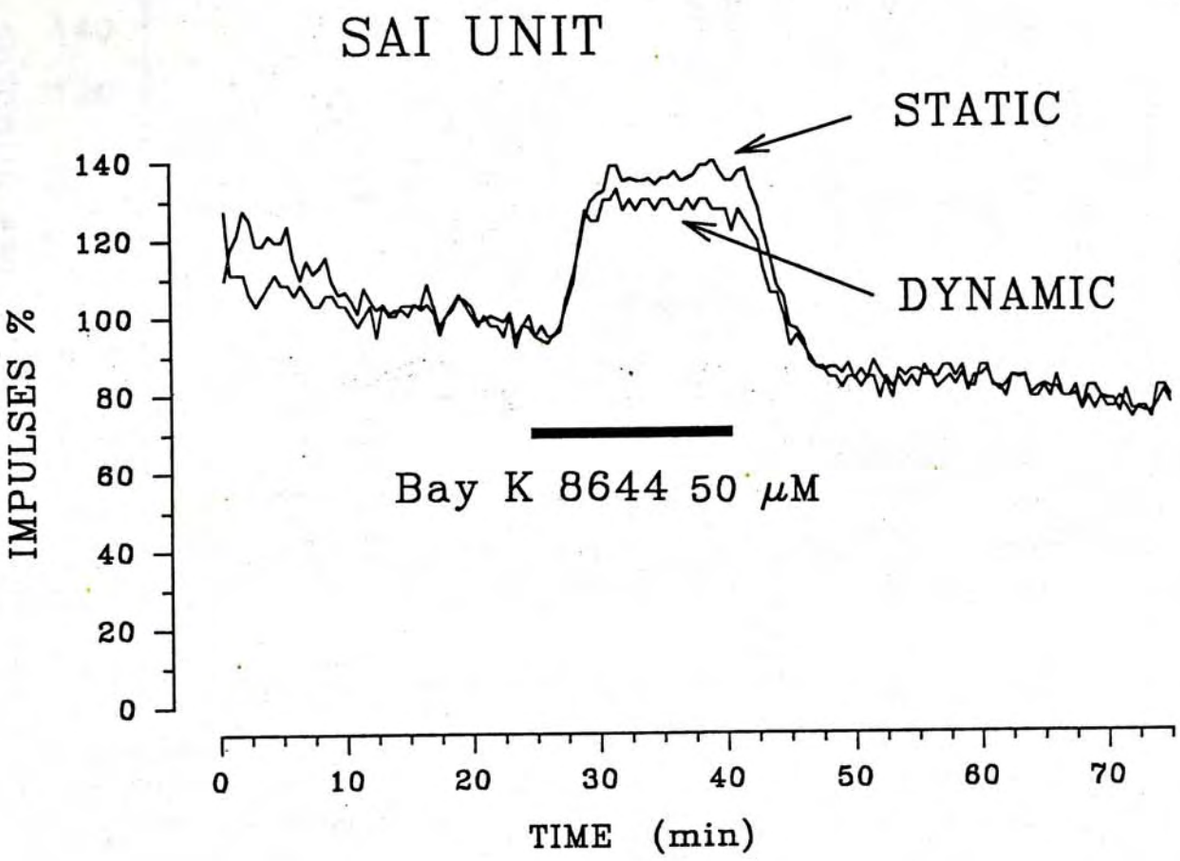


Fig 3.5.2 Effects of Bay K 8644 on static and dynamic response of a SAI receptor.

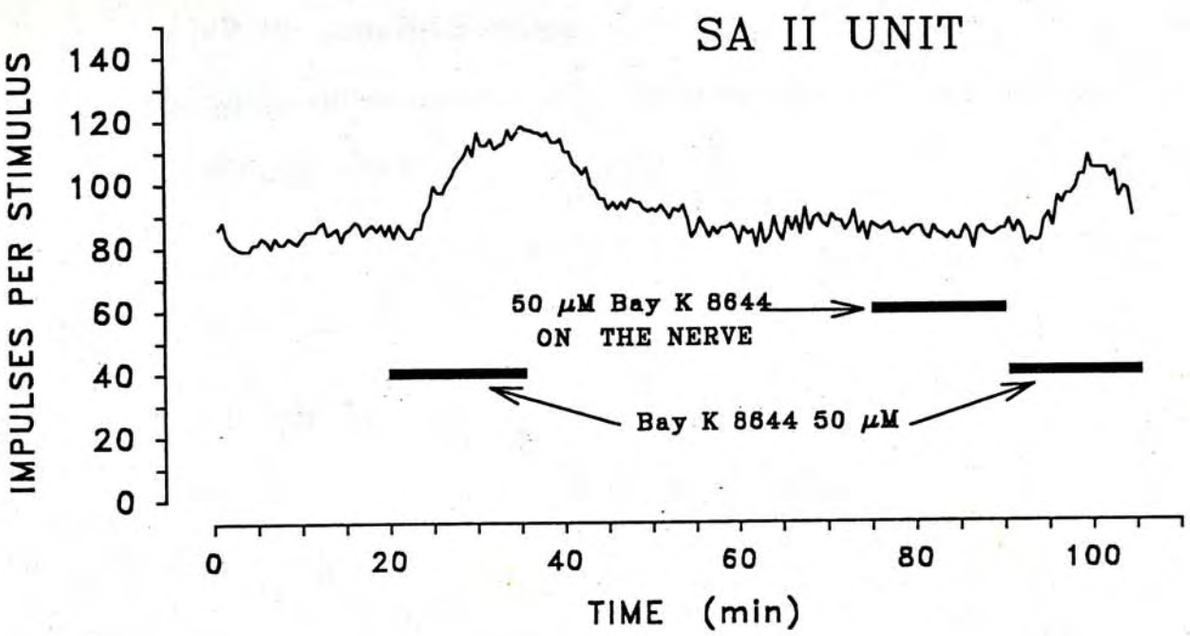


Fig 3.5.3 Effect of Bay K 8644 on a SAII receptor. Before the second application to the receptor Bay K 8644(50μM) was perfused through the nerve chamber for 15 minutes while the receptor was superfused with normal SIF.

3.6 Effects of Caffeine

In muscle caffeine increases the release of calcium ions from intracellular stores. It is also a strong inhibitor of the enzymatic breakdown of cyclic AMP by phosphodiesterase. Cyclic AMP acts on the α_1 subunit of dihydropyridine sensitive calcium channels. The effect of caffeine was tested in three SA I and two SA II units.

Type I receptors

SA I units were exposed to caffeine at concentrations of 1 and 2 mM resulting in an increase in responses by 15 and 25 % respectively (Fig. 3.6.1).

Type II receptors

On SA II units, caffeine had a similar effect as type I units. In addition, the spontaneous discharge rate was also affected to approximately the same extent (Fig. 3.6.2).

Thus, caffeine increased the responses of both SA I and SA II receptors to a similar degree.

IBMX and cAMP

The caffeine analogue isobutylmethylxanthine (IBMX) and permeable cAMP analogues 8-Bromo-cAMP and CPT-cAMP were tested on two SA I and two SA II units.

IBMX (0.3 mM) had a stimulatory effect on SA I responses similar to that observed from application of 1 mM caffeine (Fig. 3.6.3) while the effect on SA II receptors appeared to be even stronger than that of 1 mM

caffeine (Fig. 3.6.4). The membrane permeable CPT-cAMP and 8-Bromo-cAMP also increased the response of SA II receptors (Fig. 3.6.5).

These preliminary results demonstrate an involvement of cAMP in the function of both SA I and SA II receptors. The effects of caffeine were likely to have been mediated through cAMP acting on calcium channels in the cell membrane. With our present method it was not possible to detect any additional effects of caffeine on the release of calcium from intracellular stores.

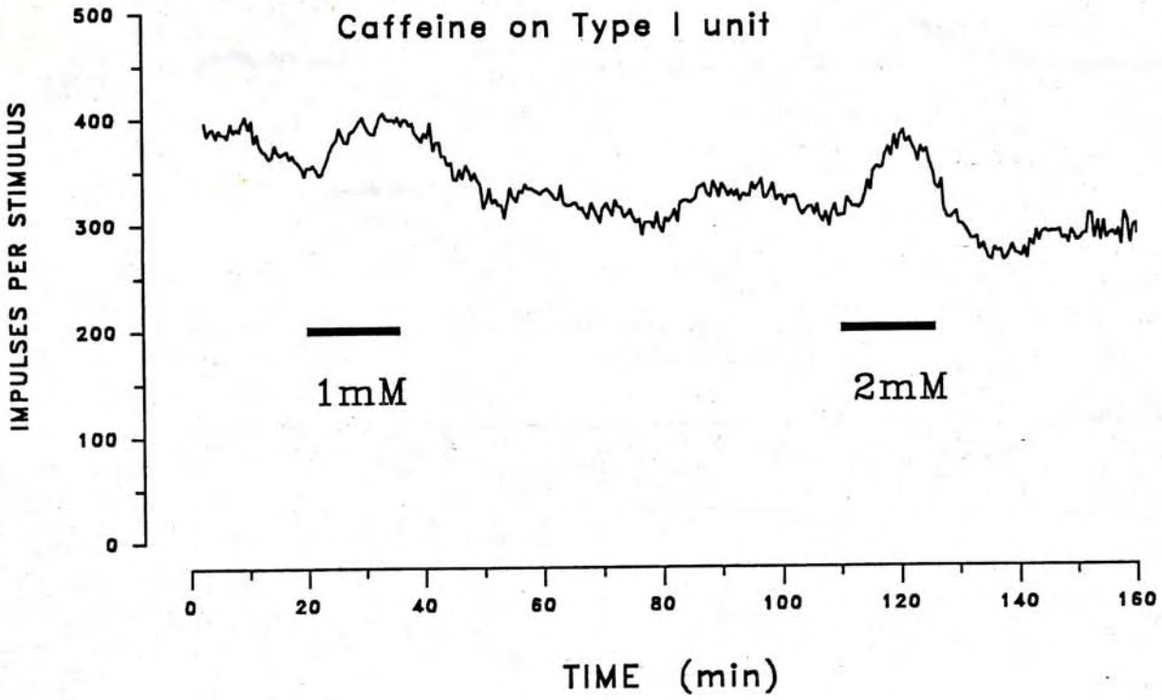


Fig 3.6.1 Effect of caffeine on type I receptor.

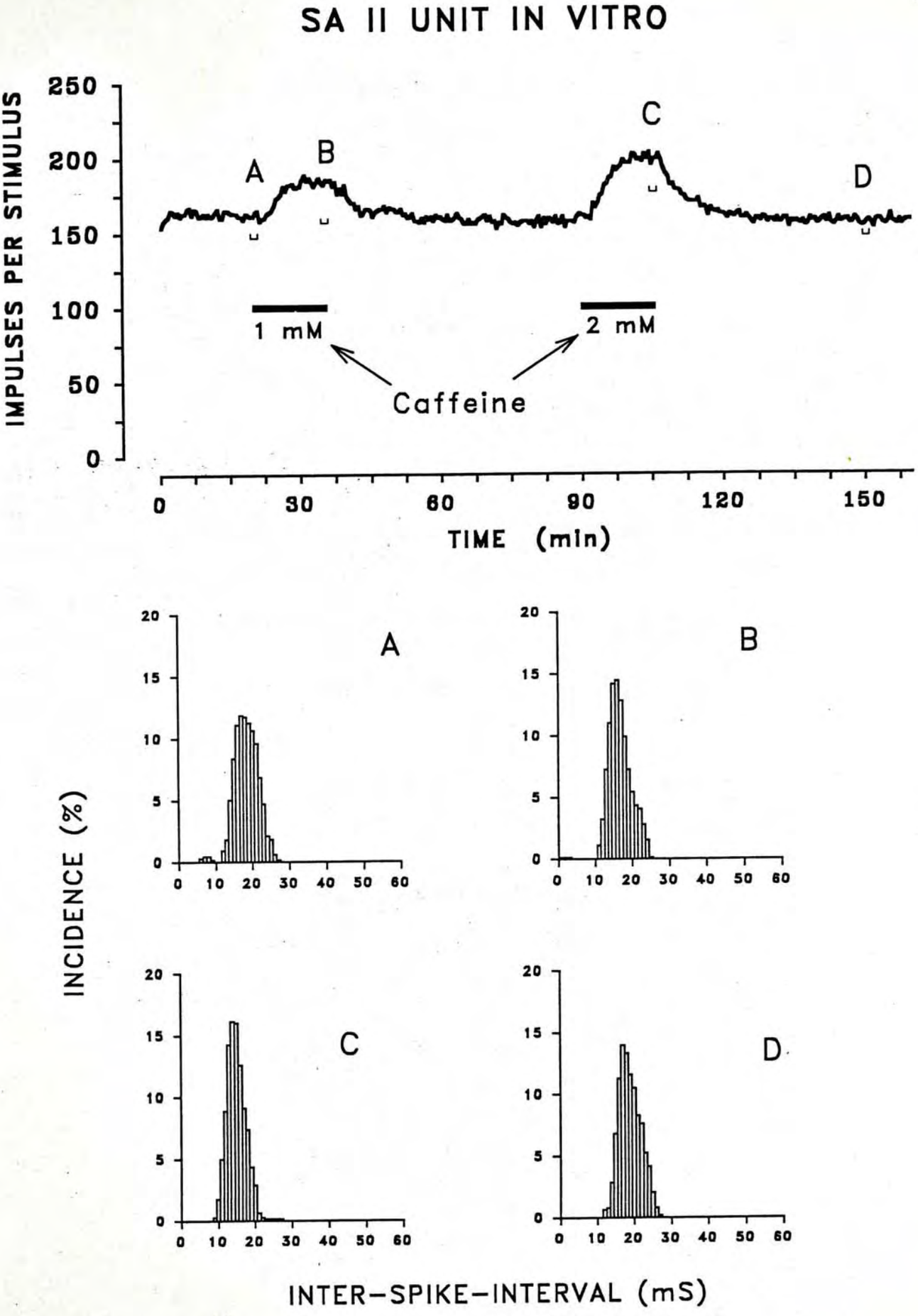


Fig 3.6.2 Effect of Caffeine on SAII unit.

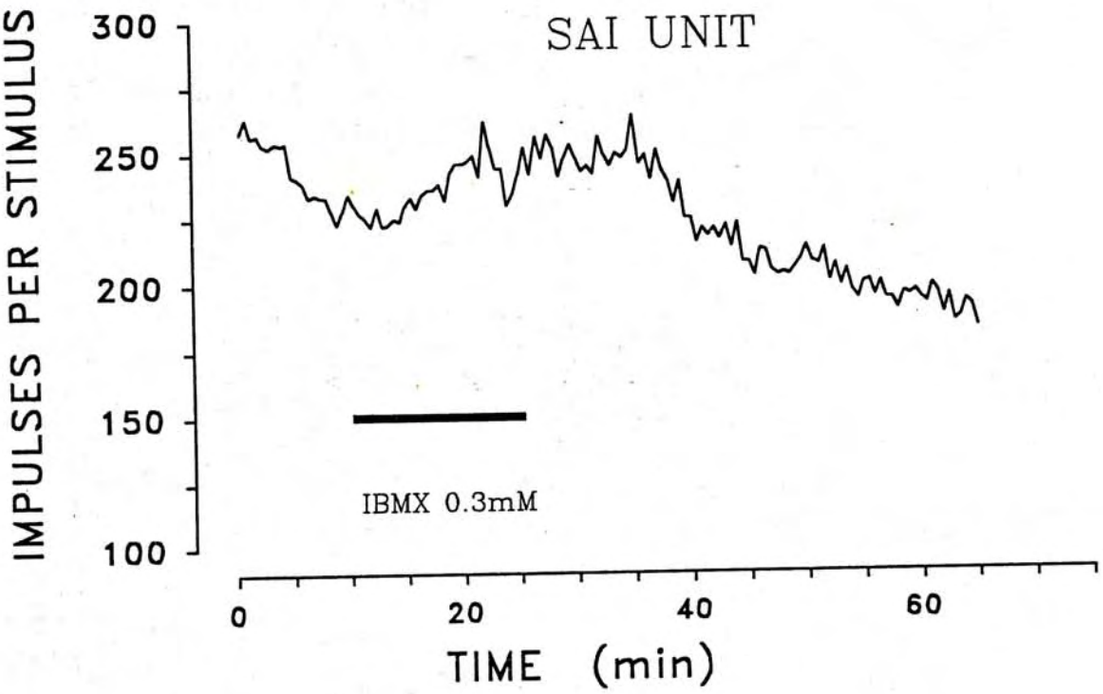


Fig 3.6.3 Effect of IBMX on SAI unit.

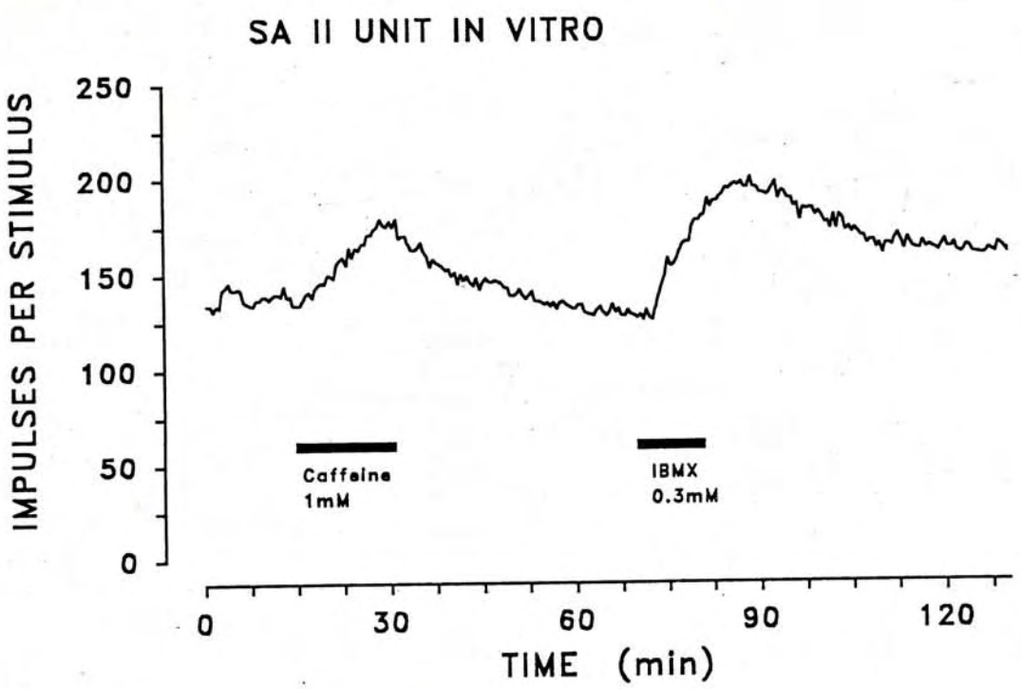


Fig 3.6.4 Effect of Caffeine and IBMX on SAII unit

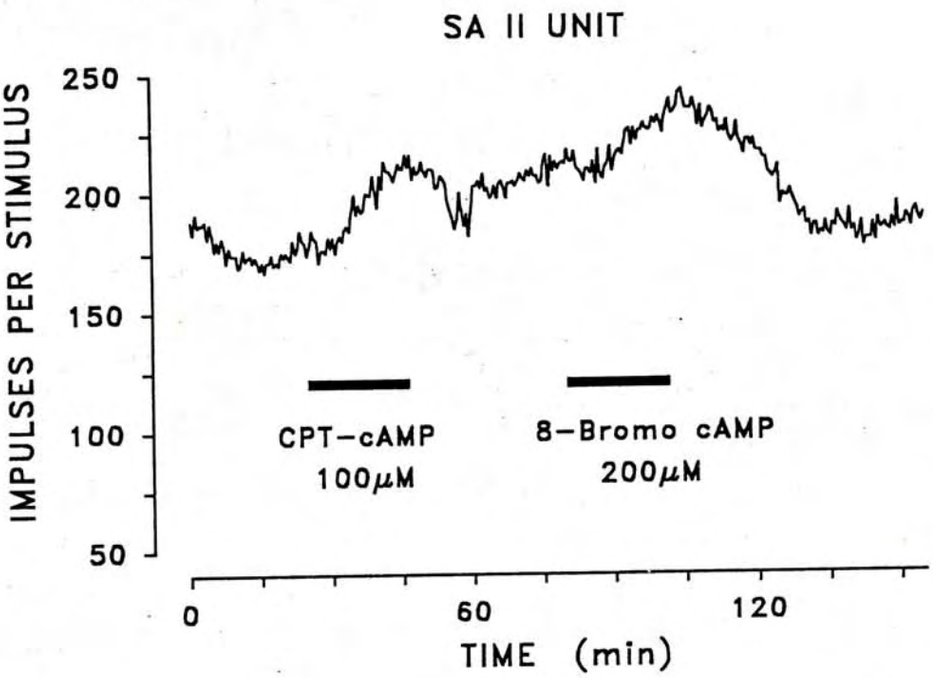


Fig 3.6.5 Effect of CPT-cAMP and 8 Bromo-cAMP on SAII receptor.

3.7 Effects of Sodium Azide

Sodium azide blocks cytochrome a in mitochondria, thus blocking the energy supply for the sequestering of free intracellular calcium into mitochondrial stores. The effect of sodium azide was tested in six SA I and two SA II units.

Type I receptors

Sodium azide had a strong inhibitory effect on SA I receptors (Fig. 3.7.1). Responses were reduced by 50 % after perfusion of the receptor sites for 15 minutes with 1 mM NaN_3. Receptors were able to quickly and completely recover upon returning to the normal solution. Higher concentrations of NaN_3 (3 mM and 5 mM) could reduce the responses further, down to 10 % of the original level (Fig. 3.7.2). It should be pointed out that receptor responses recovered well within about 10 minutes after returning to normal solution. The ISI histograms during superfusion with sodium azide appeared flat, intervals were scattered widely and did not form multiple peaks (Fig. 3.7.3).

Type II receptors

The responses of SA II receptors were increased by about 25 % when they were superfused with 1 mM sodium azide (Fig. 3.7.4) and returned to the original level when the sodium azide was washed away.

Nerve Conduction

When sodium azide was applied directly on to the nerve through the nerve chamber perfusion while the receptor site was superfused with the normal solution, receptor responses remained unchanged (Fig. 3.7.5) indicating that sodium azide did not affect nerve conduction.

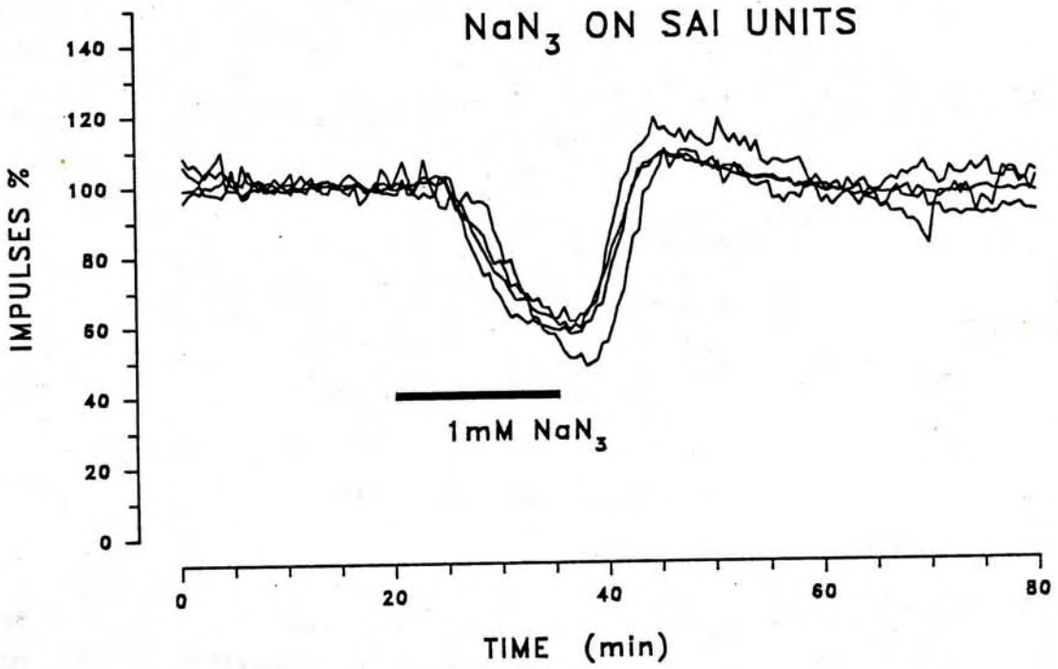
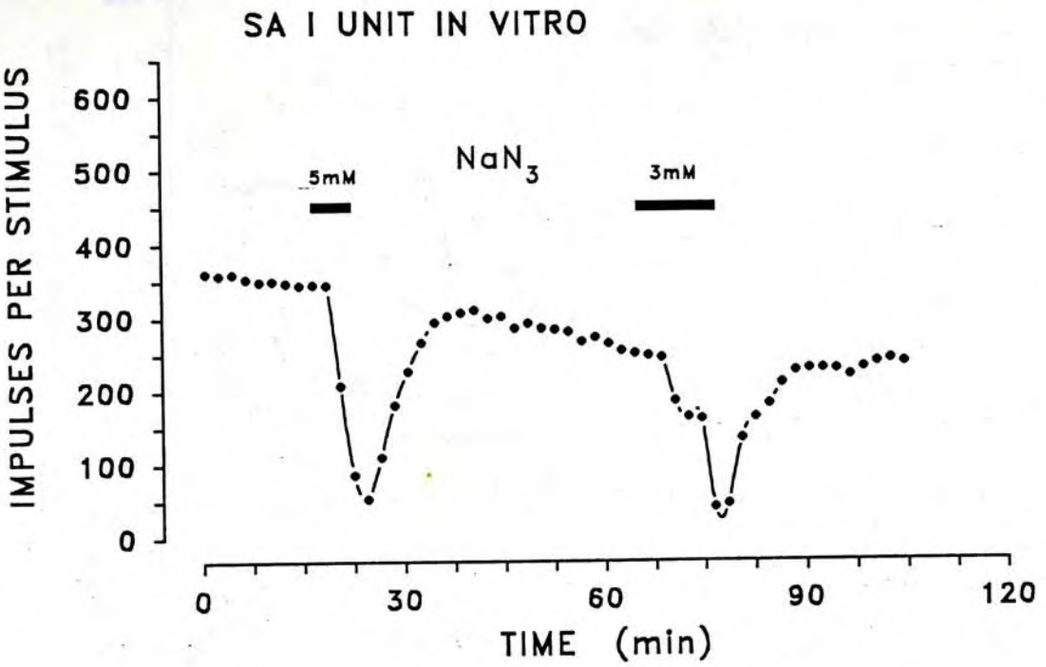


Fig 3.7.1 Effect of 1mM NaN3 on SAI receptors (n=4)



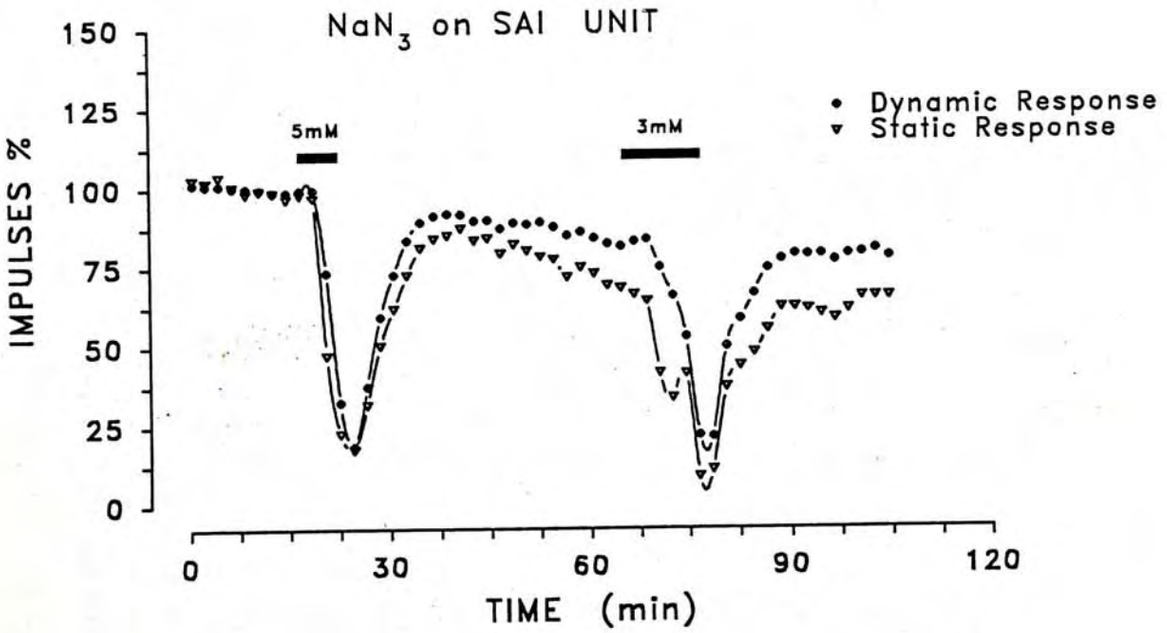


Fig 3.7.2 Effects of High concentration of NaN_3 on SAI receptor.

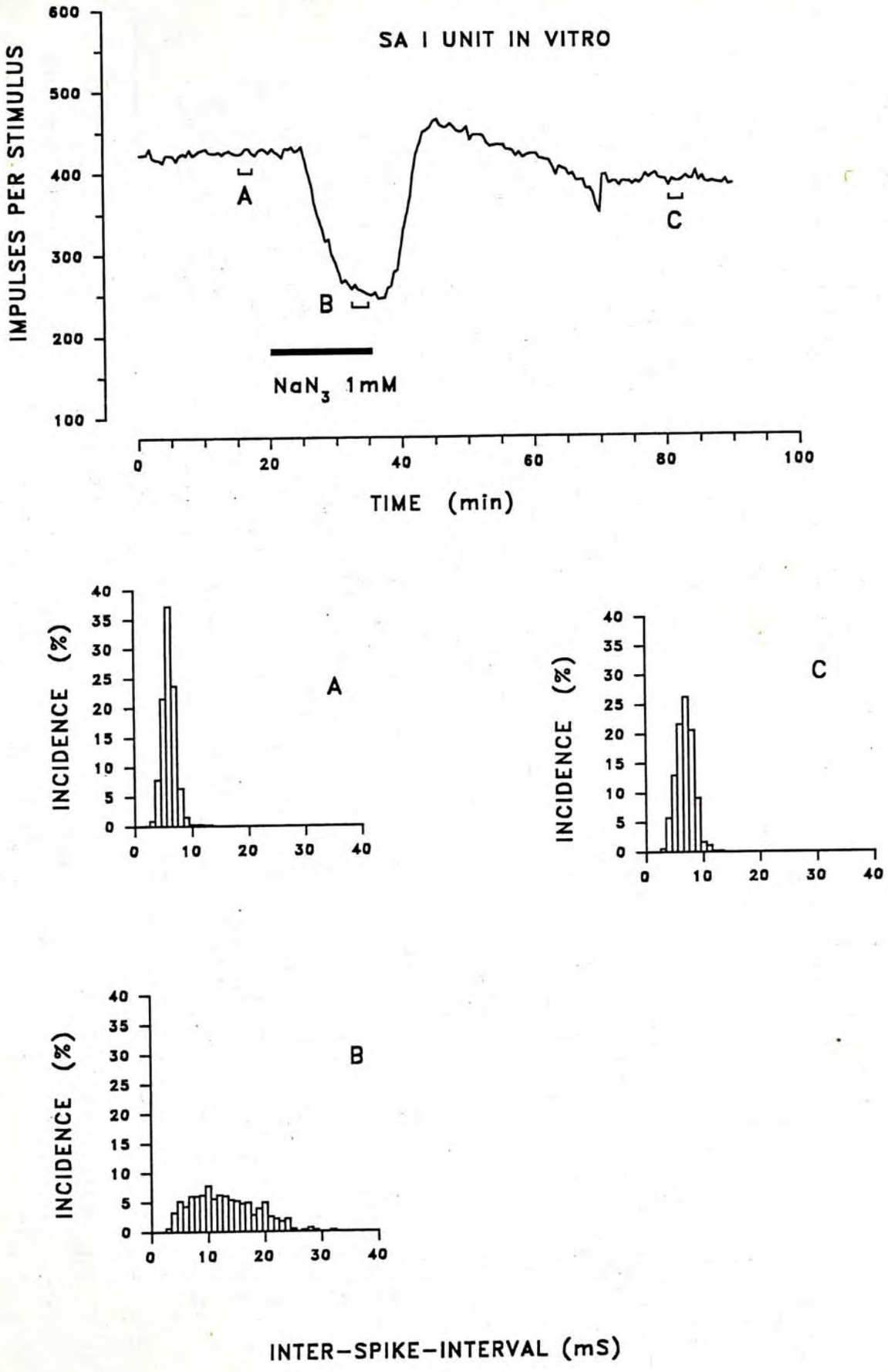


Fig 3.7.3 Effect of NaN_3 on the discharge pattern of SAI receptor.

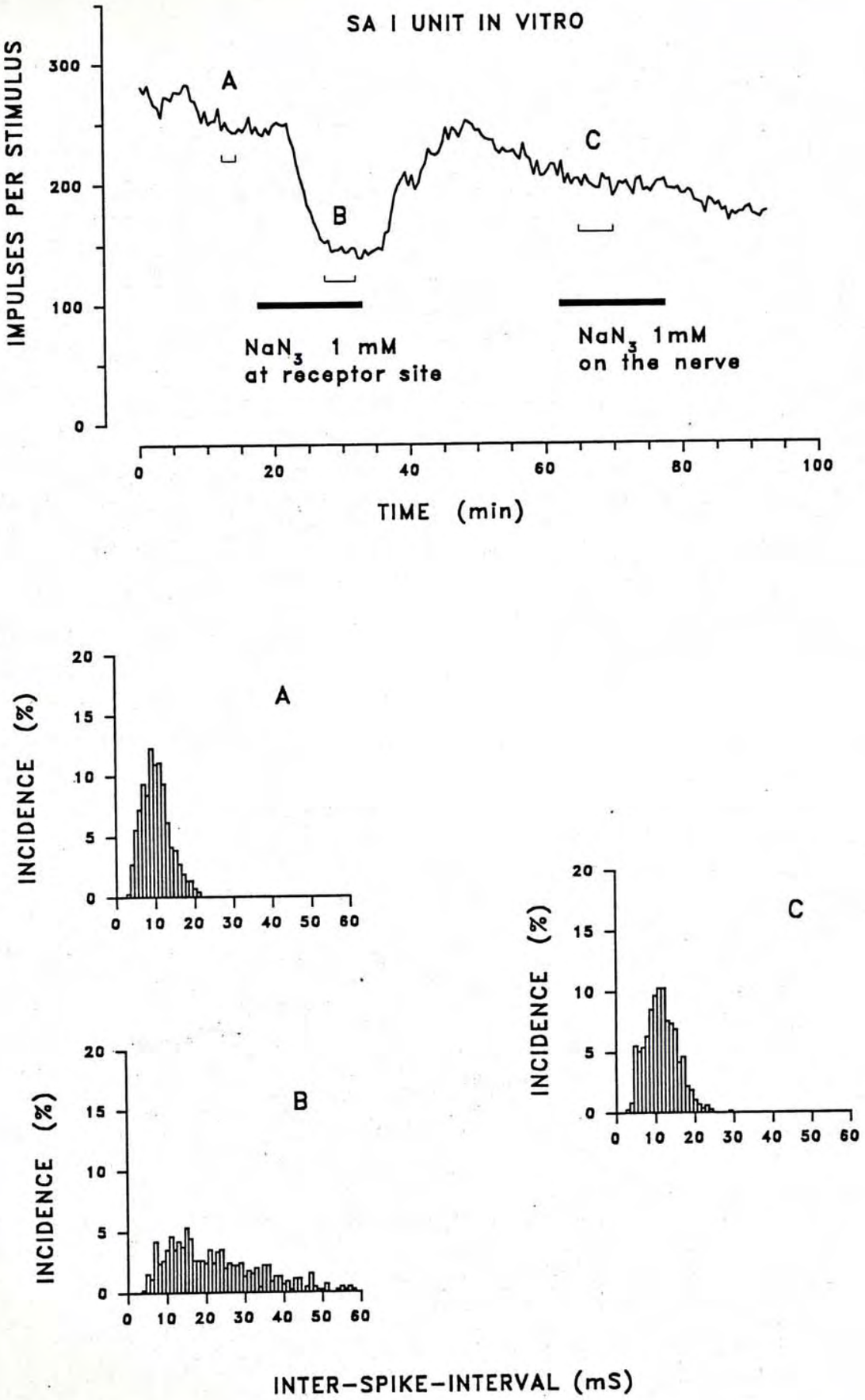


Fig 3.7.4 Effect of NaN_3 on SAI unit when perfused at the receptor site and on the nerve.

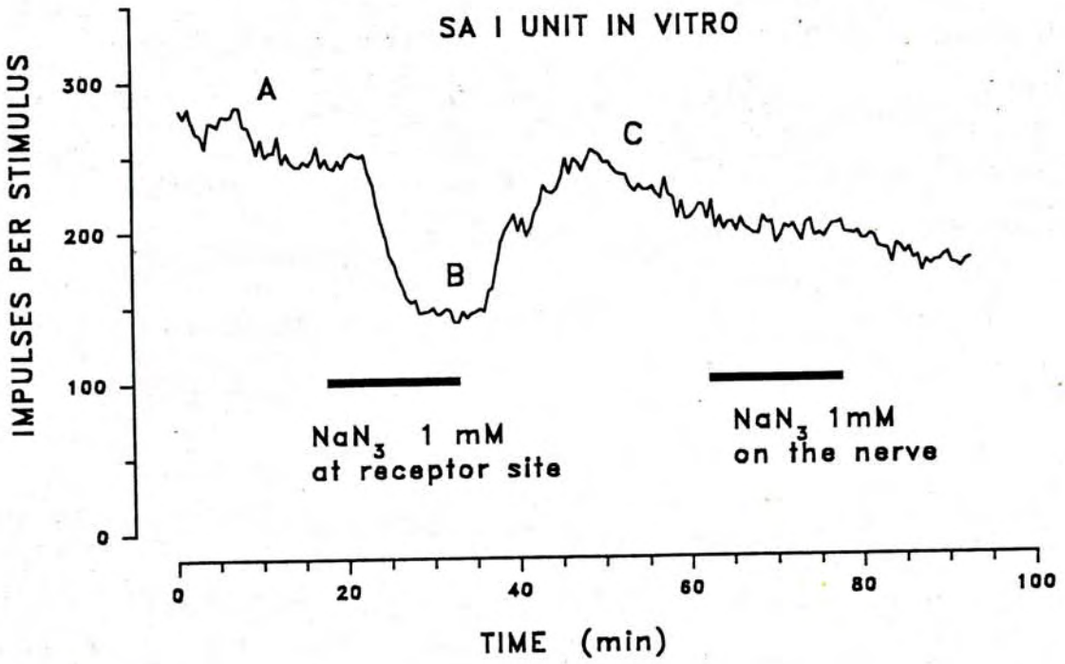


Fig 3.7.5 Effect of NaN_3 on SAI unit when perfused at the receptor site and on the nerve.

SECTION 4. DISCUSSION

4.1 Reliability of data obtained from the present in vitro preparation

The present experiments were carried out in an isolated skin-nerve preparation originally developed by Reeh (1986) and modified in this laboratory for investigations on slowly adapting mechano-receptors. The two receptor types of special interest in this study namely the slowly adapting type I and type II (SA I and SA II) receptors were easily identified by their slowly adapting firing of action potentials in response to maintained mechanical stimulation. Type I receptors characteristically responded to stimulation of discrete spots of the skin with the typical irregular discharge pattern (Horch et al., 1974) while type II receptors displayed ongoing discharge in the absence of mechanical stimulation which also increased during stretching of the skin and displayed a rather regular discharge pattern (Chambers et al., 1972). In this preparation type II receptors were much more difficult to find than type I receptors. This is not surprising as they are usually located deeper in the subcutaneous tissue layer (Andres & v.Düring, 1973) where the dissection process of the isolated skin had to be carried out. Thus, only the superficially located type II receptors could be isolated in functional order in this in vitro preparation.

Discharge rates of the receptors investigated in this in vitro study compared well with those obtained in previous in vivo experiments in this laboratory using the same type of mechanical stimulation (Baumann et al., 1990). Furthermore, receptor responses were found to be stable over more than 5 hours of superfusion with repetitive stimulation every 30 s (Baumann & Tsu, 1990) decreasing only by about 5 % per hour. In isolated preparations, a number of problems may arise related to the rather

small oxygen carrying capacity of saline based solutions in comparison with blood and the gradual development of tissue oedema (Reichel, 1976). Various investigators have tried to overcome these problems through reducing the oxygen demand by operating at temperatures far below the physiological range. The present isolated skin-nerve preparation appeared not to be affected by such problems as the oxygen consumption of the skin is lower than of most other organs and the skin temperature is already physiologically well below 37°C. In consequence, in this preparation kept at 28°C the oxygen demand was adequately met by the supply through superfusion with (5 ml/min) synthetic interstitial tissue fluid (SIF; see Bretag, 1969) bubbled with 95 % O_2 and 5 % CO_2 in a column of 1.5 m height resulting in stable receptor responses over several hours. Residual indentation of the skin at contact force of 0.5 mN and maximal indentation required to maintain the stimulation force of 15 mN were measured for each stimulus throughout the experiments. No systematic trend of changes in these displacement parameters, which would be indicative of developing skin oedema, was observed in the present experiments.

4.2 Comparison of the role of calcium in SA I and SA II receptors

The present experiments were designed to compare the effects of interfering with transmembrane currents of calcium ions in type I and type II mechanoreceptors in order to elucidate similarities and differences which may lead to a better understanding of the role of Merkel cells in the mechano-electric transduction mechanism of SA I receptors. No specialized cells are found in SA II receptors (Ruffini corpuscles) and the mechano-electric transduction process occurs directly in the fine endings of the afferent nerve fibre without involvement of a synapse (Chambers et

al., 1972). In contrast, there is still controversy about the function of Merkel cells in SA I receptors as reviewed in section I of this thesis. It has been postulated that the Merkel cell acts as mechano-electric transducer and transmits the information through a chemical synapse to the afferent nerve terminal (Iggo & Findlater, 1984). Like in most other synapses studied so far (Augustine et al., 1987) the release of transmitter substance would be controlled by pre-synaptic calcium influx (Pacitti & Findlater, 1988). Thus, drugs known to block or enhance calcium currents would be expected to affect responses of SA I receptors more pronounced than those of SA II receptors.

In previous studies in this laboratory, differential sensitivity between the two types of receptor towards the aminoglycoside antibiotic neomycin had been observed (Baumann et al., 1990). This was believed to be the result of blocking of pre-synaptic calcium channels by neomycin as observed in the neuromuscular junction by Fiekers (1983). In the present study, similar differences could only be found at a neomycin concentration of 0.5 mM. Responses of SA I receptors were reduced to about 30 % of their control responses while SA II receptors maintained about 60 % of their original responsiveness. In those experiments in situ, the concentration of neomycin was not well under control but estimated to be in the range of 0.5 to 1 mM. Responses were reduced to about 30 and 70 % of their control responses for SA I and SA II receptors respectively which is comparable with the findings of this study. However, such differential sensitivity to neomycin could no longer be observed when a concentration of 5 mM was applied to the receptors. Responses of both SA I and SA II receptors were severely suppressed by 5 mM neomycin within about 10 minutes of application (Baumann and Tsu, 1991).

Thus, in the present in vitro experiments achieving better control of the concentration of neomycin at the receptor site, the differential effect on responsiveness of the 2 types of receptor depended strongly on the concentration of neomycin. At a neomycin concentration of 0.5 mM the difference was similar, possibly not quite as pronounced as in the in situ studies. In contrast, at the higher concentration of 5 mM type II receptors appeared to be more or at least equally suppressed as type I receptors. Thus, while the dose response curve for the effect of neomycin on SA I receptors was flat in the range of concentrations examined in these studies it appeared to be steep for the corresponding effect on SA II receptors. Therefore, discrepancies between the in situ and in vitro studies might be the result of differences in the effective concentration of neomycin at the sensitive sites in the receptors.

In isolated cochlear outer hair cells from the guinea pig, neomycin in a concentration of about 50 μM was found to reduce the influx of calcium ions caused by depolarization of the cells to half the control value without major effect on the motility of these cells (Dulon & Aran, 1989). It was therefore suspected that the effect of neomycin on Merkel cell receptors may be the result of blocking calcium channels in the cell membrane and interfering with a step in the mechano-electric transduction process which exists only in type I but not in type II receptors (Baumann et al., 1990). This assumption has led to the present study examining the effects of more specific calcium channel blocking agents on both types of receptor.

Magnesium ions are known to block L-type calcium channels (Wu & Lipsius, 1990). In slowly adapting mechanoreceptors in the frog, Mg^{++} (10 to 20 mM) was found to suppress responses of type I

receptors stronger than those of type II receptors (Yamashita et al., 1986). In the present study, Mg^{++} reduced the responses of the two types of receptor to a similar degree. For both receptors, concentrations of $\geq$5 mM Mg^{++} were necessary to produce a clear effect on responsiveness. The shape of the ISI histograms was not significantly altered in either type of receptor during the time of reduced responsiveness under magnesium. Therefore, it is likely that magnesium affected both SA I and SA II receptors through the same mechanism. Similar to the observations in frog type II receptors (Yamashita et al., 1986) in the present study ongoing activity of the SA II receptor was affected to a much greater degree than the responses to mechanical stimulation. This might indicate an additional effect on the receptor threshold. An increased calcium concentration in the superfusing solution did not antagonize the effect of Mg^{++}. On the contrary, receptor responses dropped further. Doubling of the Ca^{++} concentration alone could similarly reduce the responses of SA I receptors. Such an effect might be caused by the screening effect of surface charges through the increased number of Mg^{++} or Ca^{++} ions (D'Agrigo, 1973; Green & Andersen, 1991). External calcium is well known to be important in maintaining stability of excitable membranes mainly through the screening effect.

Dihydropyridines like verapamil and BAY K 8644 are both known to affect L-type calcium channels. Calcium channels showing the characteristics of L-type channels had been identified in isolated rat Merkel cells (Yamashita et al., 1991) and might be important for the putative release of transmitter substances from Merkel cells through exocytosis of dense-core vesicles. In line with observations on L-type channels in other tissues, BAY K 8644 and cAMP increased the responses

of SA I receptors. However, the same increase in responses was observed in the present study for SA II receptors. Furthermore, verapamil in concentrations up to 10 μM had no effect on responses of either type of receptor. L-type channels found in most other tissues were blocked by nanomolar concentrations of verapamil. In studies on pre-synaptic calcium currents, two currents with different activation and inactivation characteristics were found. Only the slow component was blocked by dihydropyridines while the fast component believed to initiate transmitter release was unaffected (Penner & Dreyer, 1986). It was suggested that these two currents represent two different subpopulations of Ca^{2+} channels (Yawo, 1990; Lemos & Nowycky, 1989). Thus, the present finding that responses of type I receptors were not suppressed by verapamil in commonly used concentrations does not exclude the involvement of synaptic transmission as there is still controversy about the types of calcium channels involved in synaptic transmitter release (Smith & Augustine, 1988).

However, at high concentrations of 100 μM verapamil did actually suppress receptor responses. This confirms previous observations by Pacitti & Findlater (1988) that verapamil in concentrations between 20 and 100 μM reduced responses of SA I receptors. In this study, the same effect could also be observed when only the afferent nerve was exposed to 100 μM verapamil. Therefore, the most likely cause for these observations was a non-specific block of nerve conduction, similar to the reports by Chang et al. (1988 and 1989). This effect manifested itself in the ISI histograms in the appearance of additional peaks at multiples of the original main interval which could be seen as an indication that the receptor still produced action potentials in the same way as during the

control period but conduction of e.g. every other spike was suppressed in the afferent nerve.

BAY K 8644, known to increase the opening time of calcium channels (Pruess et al., 1985), clearly had a stimulatory effect on both type I and SA II receptors. Such an effect could not be obtained when BAY K 8644 was applied to the afferent nerve alone, thus excluding the possibility that conduction in the afferent nerve fibre had been affected. Therefore, the site of action must be in the receptors themselves. The lack of differences between the two types of receptor suggests that mechano-electric transduction channels rather than channels specifically involved in the release of neurotransmitter had been affected by this drug.

Attempts to modulate the influx of calcium ions had shown some quantitative but no qualitative differences between the type I and type II receptors. This could best be explained assuming that the two receptors operate with similar mechano-electric transduction channels and have similar calcium requirements in that respect. Although calcium influx appears to be equally important in both types of receptor, differences might exist in the way how calcium is handled intracellularly.

Caffeine is known to increase the release of Ca^{++} from the sarcoplasmic reticulum of skeletal muscles (Bianchi, 1975). Caffeine is also a strong inhibitor of the break down of cyclic-AMP by phosphodiesterase (Kumbaraci & Nastuk, 1982) and may therefore affect cAMP regulated calcium channels in the cell membrane. In some tissues cAMP increases the opening probability of L-type calcium channels (Meldolesi & Pozzan, 1987). The role of cAMP in the mechano-electric transduction process of slowly adapting mechanoreceptors had not been reported previously. In the present experiments, caffeine was found to

have a stimulatory effect on both SA I and SA II receptors. In order to test whether this effect was mediated through an increase in intracellular cAMP, the effects of the phosphodiesterase inhibitor IBMX and of the membrane permeable cAMP derivates 8-Bromo-cAMP and CPT-cAMP were examined. An increase in cAMP either by slowing of the break down of endogenous cAMP or by entering the cell in permeable form increased the responses of both type I and type II receptors. Thus, the effect of caffeine on both types of receptor observed in the present experiments is likely to be mediated through cAMP sensitive membrane channels rather than through alterations in the release of calcium from intracellular stores. Similar effects of cAMP had been observed in afferent C-fibres where it produced hyperalgesia in these sensory fibres (Taiwo et al., 1989).

Sodium azide had been shown to increase intracellular calcium concentration by blocking the energy supply for the active uptake of Ca^{++} into the mitochondria of the motor endplate and thus facilitate transmitter release (Anwyl & Lee, 1983; Dubinsky & Rothman, 1991). In the present experiments, reversible dose dependent effects on the responsiveness of both types of receptor could be observed. Thus, the energy supply was only temporarily impaired and did not cause permanent damage to the receptors. When applied to the afferent nerve, no effect could be seen. In contrast to expectations, responses of type II receptors were found to increase, while responses of SA I receptors were found to decrease during application of sodium azide. Such findings cannot be explained by the assumption of an increased intracellular Ca^{++} concentration facilitating excitatory synaptic transmission between Merkel

cell and nerve fibre. Only if the synapse were of inhibitory nature could these experimental observations be easily explained.

4.3 Synaptic link between Merkel cell and afferent nerve fibre?

In previous studies in this laboratory, responses of SA I receptors were found to be more suppressed by neomycin than those of SA II receptors (Baumann et al., 1990). Similar observations were made by Yamashita et al. (1986) in frog type I and type II mechano-receptors using magnesium and manganese as calcium channel blockers. In cat and rat, Pacitti & Findlater (1988) found responses of slowly adapting type I receptors to mechanical stimulation severely suppressed when exposed to verapamil, Cd^{2+} or Co^{2+}, while responses to electrical stimuli remained unchanged. It was therefore speculated that the mechano-electric transduction process in type I receptors involves a step which does not exist in type II receptors. This step was assumed to be synaptic transmission between Merkel cell and afferent nerve fibre. Drugs interfering with pre-synaptic calcium currents as shown for neomycin by Fiekers (1983) and Suarez-Kurtz & Reuben (1987) would thus impair synaptic transmission. The recent identification of L-type calcium channels in Merkel cells by Yamashita et al. (1991) added further support to this notion.

In order to systematically test this hypothesis in mammalian Merkel cell receptors, SA I and SA II receptors had been subjected in this study to a number of substances known to affect the influx of calcium ions into cells. The previously observed differential effect of neomycin on the responses of the two types of receptor was found to depend strongly on the concentration when re-examined in the present study with better

control of drug concentrations. Reduced calcium influx in the presence of magnesium ions suppressed responses of both types of receptor to a similar degree. Previously reported findings (Pacitti & Findlater, 1988) that the classical L-type calcium channel blocker verapamil impaired responses of type I receptors could not be confirmed when "physiological" concentrations of this drug were applied. However, at concentrations of 50 and 100 μM verapamil could impair nerve conduction mimicking suppression of receptor mechanisms. The special design of the present experimental set-up allowed to distinguish between effects on mechano-electric transduction mechanisms in the receptors and effects on conduction of action potentials in the afferent nerve.

The present results confirm previous findings that calcium currents appear to be important for normal functioning of both SA I and SA II receptors. However, a clear separation between pre-synaptic calcium currents and that part of the mechano-electric transducer current carried by calcium ions could not be established. The increase in responses of SA I receptors in the presence of BAY K 8644 and during increased intracellular levels of cAMP would support the assumption that L-type channels are involved. The lack of effect of verapamil on these calcium channels cannot be interpreted as an argument against the hypothesis that these channels are involved in the release of neuro-transmitter. Verapamil resistant calcium channels likely to initiate transmitter release in the motor end plate had been observed by Penner & Dreyer (1986) suggesting differences from the classical L-type channels.

Increased calcium influx under the influence of BAY K 8644 or increased intracellular levels of cyclic AMP (application of caffeine, phosphodiesterase inhibitors or membrane permeable cAMP) resulted in

increased number of spikes in both SA I and SA II receptors. This would suggest that in both receptor types calcium ions are also involved in the transducer currents through mechanically gated channels similar to findings in isolated hair cells of the inner ear (Ohmori, 1985; Kroese et al., 1989). In the present study no separation was achieved between possible effects on that part of the mechano-electric transducer currents carried by calcium ions and the putative pre-synaptic calcium currents controlling neuro-transmitter release as seen in various other synapses (Augustine et al., 1987).

However, qualitatively different effects were observed under the influence of sodium azide. This substance blocks cytochrome A, thus impairing the sequestration of calcium into mitochondrial stores. The resulting increase in free intracellular calcium concentration has been demonstrated to increase the release of neurotransmitter (Anwyl & Lee, 1983; Dubinsky & Rothman, 1991). According to the findings at the neuromuscular junction by Anwyl & Lee (1983) increased responsiveness of SA I receptors was expected. However, the opposite effect was observed which could only be explained by the assumption of an inhibitory synapse between Merkel cell and nerve terminal.

Attempts to interfere with intracellular calcium stores which might be involved in the control of synaptic transmitter release did so far not support the hypothesis of excitatory synaptic transmission between Merkel cell and afferent nerve terminal. But further studies need to be carried out involving different approaches to interfere with intracellular calcium stores and possibly direct fluorometric measurements of intracellular calcium concentrations in Merkel cells.

Thus, although the results of the present study do not provide support for the hypothesis of the Merkel cell being the mechano-electric transducer and acting via an excitatory synapse on the afferent nerve terminal they do neither provide conclusive proof against this hypothesis. The finding of met-enkephaline like substances in Merkel cells by Hartschuh et al. (1979) and Cheng Chew & Leung (1991) would certainly not contradict the assumption of an inhibitory synapse as met-enkephalines are mostly found in inhibitory synapses. However, this would still leave the question open which part of the Merkel cell neurite complex acts as the mechano-electric transducer proper. It could be speculated that the met-enkephalins might act as inhibitory neuromodulators on the release of another excitatory substance from the Merkel cells or on the generation of receptor potentials in the nerve terminal.

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